Role of mouse motor cortex in the behavioral response to unpredictable visual feedback

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Thank you.

2. SUMMARY

«The role of the motor system is to produce movement, not to describe it.» **(Paul Cisek)**

Movement is the way by which we interact with the world. This often only becomes apparent in case of motor apparatus dysfunction such as in neurological conditions of movement disorders highlighting how seemingly effortless the nervous system performs movement generation. Moreover, many animal species are capable of moving in a stunningly vast array of ways which underscores the requirement for sophisticated degrees of control. Furthering insight into motor control therefore underlies a fascination for the complexity of the nervous system as well as a substantial medical interest.

To understand the role of motor cortex, a high-level brain area, in the control of sensory guided movements, I first trained head-fixed mice to navigate a two-dimensional virtual reality environment with occasional perturbations of the expected visual feedback. During learning, I used activity manipulation by optogenetics to understand the involvement of motor cortex in active behavior. Additionally, using activity recording by two-photon calcium imaging in genetically defined projection cell types, I probed for neuronal activity patterns potentially mediating this behavior.

I found that motor cortex is a critical brain area for execution of visually guided behavior which mediates the learning of the navigation task. Activity recordings yielded a myriad of neuronal responses correlating with the mouse behavior, often multiplexed within the same cell. Critically, activity patterns during spontaneously executed, expected behavior differed from those during reactive behavior induced by unexpected perturbations of the virtual environment. These differences in neuronal activation could underlie the behavioral effects observed during optogenetic activity manipulations. Finally, I observed cell type-specific, learning-related changes. Notably, presumed motor output mediating cells increased in activation as mice became more efficient at executing the task.

I discuss these findings in the context of recent theories of brain function suggesting that the nervous system not only predicts the dynamics of the subject's environment but also generates movement in reaction to future predicted body states.

3. INTRODUCTION

3.1 Movement generation by the central nervous system

In most animals, movement generation is the means by which to interact with the world and it is therefore reasonable to assume that much of the nervous system is dedicated to the shaping of this process. Movement generation is achieved by the skeletal musculature which requires that motor neurons become active and inactive in a coordinated manner (**Figure 1**). That raises the question what ultimately determines the activation of these neurons which can integrate input from as much as 150 000 synapses.

In mammals, motor neurons reside in so-called motor neuron pools at distinct levels of the spinal cord in the ventral horn and are surrounded by a myriad of spinal interneurons the function of each of these classes defined by their connectivity or developmental origin has only recently begun to be understood (Stepien and Arber, 2008). For example, spinal interneurons located in the dorsal horn of the spinal cord may receive input from proprioceptors which continuously monitor the current state of the muscles and provide feedback. Additionally, supraspinal centers provide a stream of input to all segments of the spinal cord, the connectivity and precise postsynaptic targets of which are still largely unknown. Nuclei located in the reticular formation in the brainstem are speculated to provide the main go signal to execute a motor program.

The delineation of the motor program likely engages the entire brain: Changes of the

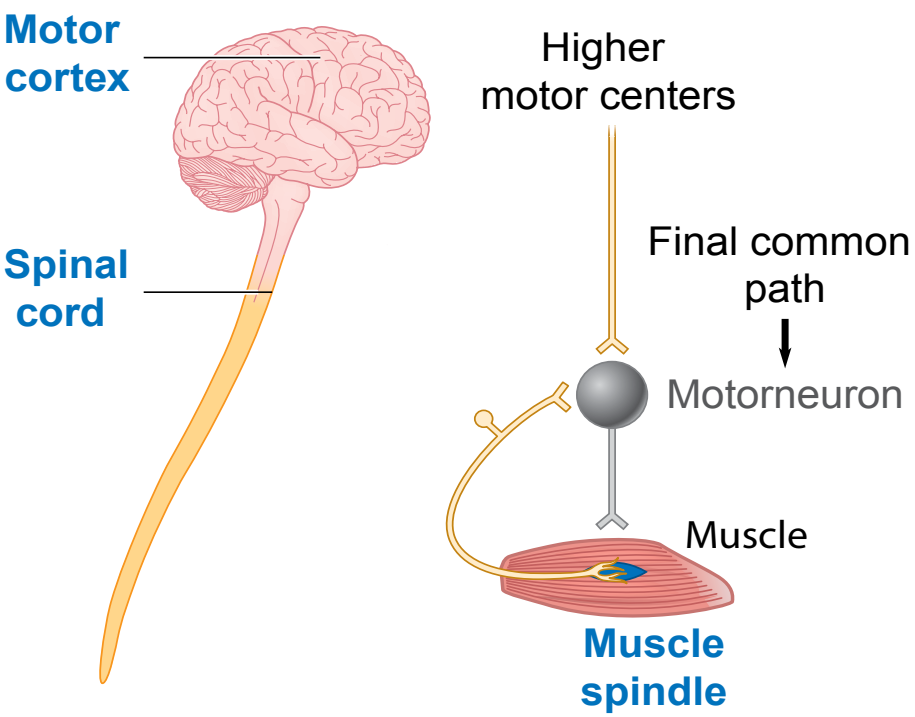

Figure 1. Generic motor control pathway

Motor cortex, the brainstem (not pointed out) and spinal cord are major motor control regions (left) whose activity critically influences motor neurons controlling muscle tension (right). Adapted from Alstermark and Isa, 2012.

environment are extracted by sensors in the periphery, routed to dedicated cortical areas where they are processed in a way which presumably shapes the ongoing and upcoming motor program. Numerous interception points allow different information streams to inject their content into this process. For example, the cerebellum likely contributes an internal representation of the environment and information on how muscles and limbs should behave given a certain exerted force. Gated by striatal circuits which also process motivational and memory-related information, motor cortex is hypothesized as the motor command issuing structure. Its activity is further finessed by reticulospinal circuits to generate appropriate motor behavior.

3.2 A historical perspective on motor cortex

The ability to generate movement is one of the defining factors of the animal kingdom. While this fact was acknowledged early on in the 18th century, the search for a substrate within the body which would instruct movement was a highly controversial one. The spinal cord was soon appreciated to take part in movement generation but the role the brain and in particular its cortex plays was called into question. A common view in the mid-nineteenth century assumed that the cerebral hemispheres would be unexcitable by many stimuli which included, in “dogs, rabbits, and goat kids” irritating the cortex with a scalpel and applying acids (Fritsch and Hitzig, 2009). Many of these experiments seem brutal and maybe not even sufficiently substantiated. Still, the controversy researchers were dealing with in former days is not fully settled today.

Sequential decerebration experiments were performed in birds by French physiologist Marie-Jean-Pierre Flourens (Pearce, 2009). He found that decerebrated animals had to be challenged in order to execute movements, they appeared “sunken in themselves” or would not change behavior even if “starved and placed on a heap of food”. In partly decerebrated animals he observed recovery without obvious impairment which led him to conclude that there would not be a dedicated site for perception. Accordingly, he didn’t believe in the concept of cortical localization whereby functions would map onto areas of the brain. However, people wondered how it could be possible that single extremities either in humans or animals could be paralyzed and still no site for control of this movement in the brain was found yet. Was there any such site beyond the spinal cord?

In an effort to demonstrate the role of the brain in movement generation, Hitzig and Fritsch reported 1870 electrical stimulation experiments in dogs (Fritsch and Hitzig, 2009). They claimed to have found a site in which movement of the dog’s legs could be reliably evoked which led to their famous statement “part of the convexity of the dog’s cerebrum is motor, another part is not motor”. This implied a functional subdivision of the cerebral surface defined by location. In support of this, David Ferrier later mapped the cortical surface of monkeys and reported defined

areas which would evoke limb movements (Ferrier, 1874). Though he didn't lay claim on any topography of movement generation, his results could have suggested such a phenomenon. Wilder Penfield 1937 repeated Ferrier's experiments in humans undergoing neurosurgery and found a similar organization of cortex (Penfield and Boldrey, 1937). Today, primary motor cortex (M1) is often defined as the area in agranular cortex in which intracortical microstimulation (ICMS) reliably evokes movements at a low threshold (rats, Brecht et al., 2004). The area adjacent and medial to M1 where stimulation was less effective was then coined secondary motor cortex (M2).

3.3 Cytoarchitecture and microcircuits

Neurons can be grouped into classes based on their morphology, their developmental origin, expression of genetic markers, their local or long-range projection patterns, their laminar position or according to their function. Likely, parameters correlate to a greater or lesser extent with each other, limiting their experimental applicability to delineate cell classes. The concept of serial homology and lamination provides a useful entry point into the classification (Harris and Shepherd, 2015). It assumes that circuit motifs subserve a similar functional role across cortical areas. Since the functional role of a cell type during behavior is assumed to be correlated with connectivity, the difference between cortical areas which bestows each region with its unique properties is hypothesized to be due to differences in precise connectivity and relative abundance of cell classes. Laminar position has been suggested to determine potential connectivity, the actual connectivity being determined by both the laminar position and long-range projection target (Anderson et al., 2010). Many of these detailing studies have been performed in mice due to the ease of genetic access in this model system which is why there is a prominent lack of knowledge regarding the cortical organizing principles in primates or humans.

Motor cortex, unlike most of neocortex, is made up of only five layers which can be further subdivided according to cellular composition, axonal projection patterns and dendritic arborization. It is believed not to contain a classic layer 4 and, because of its appearance when Nissl-stained, is also often referred to as agranular cortex. A recent report challenged this view and claimed a functional similarity of a motor cortical cell layer expressing *Rorb*, a genetic marker for layer 4 neurons in primary somatosensory cortex, to sensory cortex layer 4 neurons (Yamawaki et al., 2014).

Throughout motor cortex and cortex in general, three major, top-level, non-overlapping excitatory cell classes can be defined, the distinction of which arises largely from differential projection patterns: Intra-telencephalic (IT), pyramidal tract (PT) and cortico-thalamic (CT) neurons (**Figure 2**). All cells of these classes, which comprise about 80% of all neurons in cortex, form, to a varying degree, local recurrent connections within the same class (omitted from the figure for clarity) but the across-class probability of connection depends on the exact pre- and

postsynaptic partner. While individual motifs in sensory cortex occur with a higher frequency than chance (Perin et al., 2011; Song et al., 2005), their probability of occurrence never equals 1 which suggest that within-class subnetworks might subserve differential functions (Harris and Mrsic-Flogel, 2013).

3.3.1 Intratelencephalic projection neurons

IT neurons constitute a very diverse class of neurons found throughout the cell layers (**Figure 2**). They are the only excitatory neuron type that forms connections with the contralateral cortex by means of the corpus callosum. The lamination of their dendritic tree correlates with soma position (Morishima, 2006); their axonal arborization remains within the telencephalon.

IT neurons in layer 2/3 send a major descending projection intracolumnarly to layer 5A and layer 5B thereby exerting a powerful influence over deeper layer projection neurons (Weiler et al., 2008). In motor cortex, this projection accounts for a large fraction of the excitatory drive within the local network. Descending layer 2/3 neurons have been shown to comprise two distinct populations, one that preferentially targets crossed cortico-cortical / cortico-striatal layer

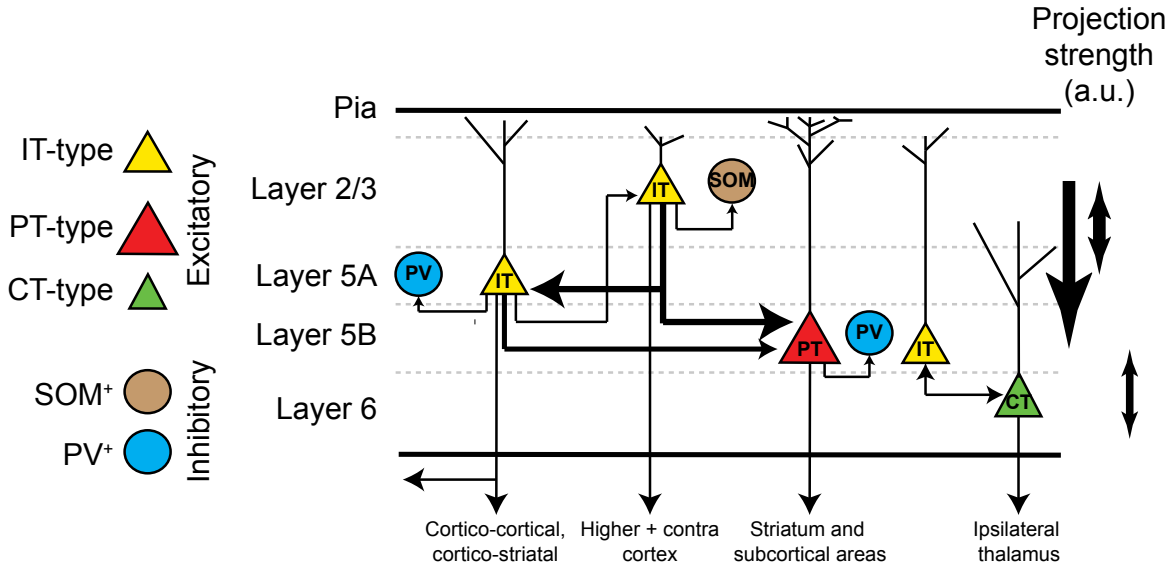

Figure 2. Local connectivity in motor cortex

Summary diagram of local connectivity as mapped by studies referred to in the main text. IT neurons provide the main translaminar, intracolumnar excitatory drive to layer 5 PT neurons which do not reciprocate the input. Local inhibitory interneurons provide laminar and translaminar inhibition. Of note, layer 5 PT neurons are the only neuron type projecting to subcortical structures. Excitatory neurons of the same type usually form extensive connections with each other which are omitted from the diagram for clarity. IT, intratelencephalic. PT, pyramidal tract. CT, cortico-thalamic. PV, parvalbumin-positive. SOM, somatostatin-positive.

5A projection neurons and a second population which preferentially targets cortico-spinal (but not cortico-striatal) layer 5B neurons (Anderson et al., 2010). The main ascending interlaminar pathway originates in layer 5A and projects to layer 2/3 (Weiler et al., 2008). Despite this strong connection, layer 2/3 cells are very reluctant to discharge *in vivo*, at least in sensory cortices, probably because of strong local inhibition (O'Connor et al., 2010). This has been suggested to be advantageous in learning models. While the IT --> PT connectivity motif is common, the reciprocal case is rarely ever found in frontal cortex suggesting a directional and hierarchical flow of information (Kiritani et al., 2012; Morishima, 2006).

In primates, IT cortico-striatal neurons have been reported to be very selective for specific movements, stimuli or contexts and thus are difficult to excite (Turner and DeLong, 2000).

3.3.2 Pyramidal tract projection neurons

PT neurons are large cells residing in layer 5B (**Figure 2**). Historically, layer 5B has been defined by the presence of PT neurons but this definition is far from exclusive as cells of the IT-type intermingle. The dendritic tree of layer 5 PT neurons extensively arborizes in layer 1 and their axons project ipsilaterally and subcortically, often to multiple target regions simultaneously. Single axons typically innervate many, but not all, potential targets including striatum, subthalamic nucleus, superior colliculus, medulla nuclei and spinal cord interneurons (Kita and Kita, 2012). Similar results have been obtained in cats (Res, 1986) and primates (Parent and Parent, 2006). Thus, in layer 5B, neighboring PT neurons can have a very distinct output matrix. Notably, PT --> IT connectivity is very scarce (Kiritani et al., 2012) which, together with the observation that the IT --> PT motif is the dominant intracolumnar excitatory input for PT neurons, implies a unidirectional flow of information. It would be of great interest to determine what role this "rectification" within the IT cell population plays and how it influences layer 5 PT neurons.

PT <--> PT connectivity is less prevalent than IT <--> IT (Kiritani et al., 2012). In primary visual cortex, neurons with similar receptive fields have been found to preferentially and strongly connect to one another (Cossell et al., 2015) in an experience-dependent manner (Ko et al., 2013). A similar set of studies has not been performed in motor cortex but it is tempting to speculate that also in motor cortex, neurons which share a similar function during active behavior are also more intimately linked together than their non-synergistic counterparts and that this link is established or strengthened upon learning new motor skills. Capaday et al. suggested, based on putative synapse mapping, in cat motor cortex a network topography in which individual neurons would bind together the representations of a number of muscles. A recent study found that in primates, the output of cortico-motorneuronal cells is organized into functions of a muscle rather than individual muscles or synergies thereof (Griffin et al., 2015). Perhaps, the selective PT <--> PT connectivity Kiritani et al. report is a reflection of PT neurons with shared functions connecting

recurrently with each other. One way to address this question is large-volume connectomic reconstruction of neurons whose activity has been previously recorded.

In the rabbit, under conditions of locomotion or postural correction PT neurons have been shown to be the cell class which was correlated with movement parameters. Cortico-cortical IT neurons and layer 6 cortico-thalamic projection neurons were reported to be much less active and suggested to be dispensable for the task (Beloozerova et al., 2003a, 2003b). Layer 5 PT neurons have also been suggested to be the output channel of cortex which drives lateralized movements (Li et al., 2015a). Activating those neurons drives contraversive licking and a larger fraction of neurons selective for contraversive movements was observed. This was not the case for layer 5 IT neurons.

Due to their diverse input (e.g. IT cells from all layers, thalamus) and output (many subcortical targets), layer 5 PT cells are suggested to integrate the results from local computations and broadcast them accordingly to distant subcortical structures (Capaday et al., 2009). The specific projection target would depend on the home area of the cell, e.g. spinal targets in the case of motor cortex or tectal targets in the case of visual cortex.

3.3.3 Cortico-thalamic projection neurons

Layer 6 CT neurons are abundant and constitute a cell class which projects to ipsilateral thalamus (**Figure 2**). Their function, especially in the behaving animal, has remained largely enigmatic. CT neurons share a pyramidal-type morphology with their dendritic tree usually not extending beyond deep layer 2/3.

The dominant connections were found to be reciprocal CT <--> CT and CT <--> IT, though the CT --> IT innervation was restricted to IT neurons in layer 6 (Yamawaki and Shepherd, 2015). CT --> PT connectivity was found to be notably scarce. CT neurons were also shown to be effective at recruiting disynaptic inhibition. Strong, translaminar CT --> interneuron connectivity is a feature that has also been observed in visual cortex (Olsen et al., 2012). The specific IT --> PT and CT <--> IT, but not CT --> PT, connectivity suggests that these two pathways would function relatively independent from each other, possibly according to behavioral state.

3.3.4 Local interneurons

Layer 2/3 excitatory neurons employ a “sparse code”, which is usually concluded from their low firing rate (Huber et al., 2012; Komiyama et al., 2010). Inhibition plays a major role in suppressing this activity and presumably it is initiated by feed-back projections from deeper layers. Still, knowledge on interneuron connectivity in motor cortex, especially in deep layers, is sparse

(**Figure 2**).

The source of activation of inhibition is mainly intralaminar and unselective with respect to projectional identity of the target cell (Kätzel et al., 2011). Layer 2/3 was shown to be a main sink of inhibition which likely contributes to the aforementioned sparse firing rate. Excitatory input to inhibitory cells showed a remarkable difference. While layer 2/3 IT neurons excited mainly somatostatin (SOM)-positive interneurons (found earlier in sensory cortex (Kapfer et al., 2007)), layer 5 projection neurons of both, cortico-striatal IT- and cortico-spinal PT-type excited predominantly parvalbumin (PV)-positive inhibitory neurons in the same layer (Apicella et al., 2012).

In rabbits, putative layer 5 motor cortex pyramidal cells and putative interneurons discharge in an antiphasic manner during locomotion *in vivo* (Beloozerova et al., 2003b). This suggests that while excitatory projection neurons command movement, inhibitory interneurons might gate it. This is at odds with a study performed in rats which found interneurons to be engaged in the on-going movement and tightly coupled to the excitatory cells (Isomura et al., 2009). There were no such cells which would be active when the rat was not moving, suggesting that interneurons actively shape rather than gate movement. Alternatively, the activity of inhibitory neurons might serve to suppress motor programs which are not required for the task at hand.

3.3.5 Summary

The emerging picture is sufficiently detailed to guide experiments to address questions with respect to *in vivo* properties of these cell classes. If and how these cells modulate their activity under different behavioral conditions is a question that connectivity studies to date largely disregarded. The excitation / inhibition ratio (E/I ratio), the imbalance of which is suggested to be cause of major psychological disorders such as schizophrenia or autism caused presumably by dysfunction of inhibitory neurons (Markram and Markram, 2010; Nelson and Valakh, 2015; Rubenstein and Merzenich, 2003; Yizhar et al., 2011). By analogy, it is possible that an imbalance in the excitation ratio of IT and PT neurons is also contributing to major disorders (Shepherd, 2013). Therefore, it is also of clinical interest to investigate in detail the activation properties of IT and PT neurons *in vivo*.

3.4 Long-range functional connectivity of motor cortex

3.4.1 Long-range input to local motor cortex circuits

Inputs to motor cortex in their entirety haven't been comprehensively mapped but studies have been performed in subregions such as secondary motor cortex and vibrissal motor cortex, yielding a patchwork picture of the organization of long-range input into local motor cortex circuitry (**Figure 3**). Detailed input mapping is performed in anticipation of being able to link potentially unique, source-specific features with the site of origin's behavioral relevance.

Input to vibrissal motor cortex was stratified according to origin of the projection but collectively was able to excite the entire motor cortical column (Hooks et al., 2013). Orbital cortex avoided all but layer 6 where CT neurons reside. Motor-related areas such as M2 and anterior nuclei of the thalamus avoided layer 6 but otherwise targeted all layers and had monosynaptic access to layer 5 PT neurons. In contrast, nuclei from the posterior thalamic group did not innervate layer 5 PT neurons but all upper layers. Likewise, input from primary somatosensory cortex (S1) was shown to target all layers, too, but monosynaptic innervation of layer 5 PT neurons was weak (Mao et al., 2011; Petrof et al., 2015). Together, this suggests that in superficial layers 2/3 and upper layer 5A processing important for sensory guided behaviors takes place. In support of this, sensory responsiveness has been observed in these layers (Huber et al., 2012; Murray and Keller,

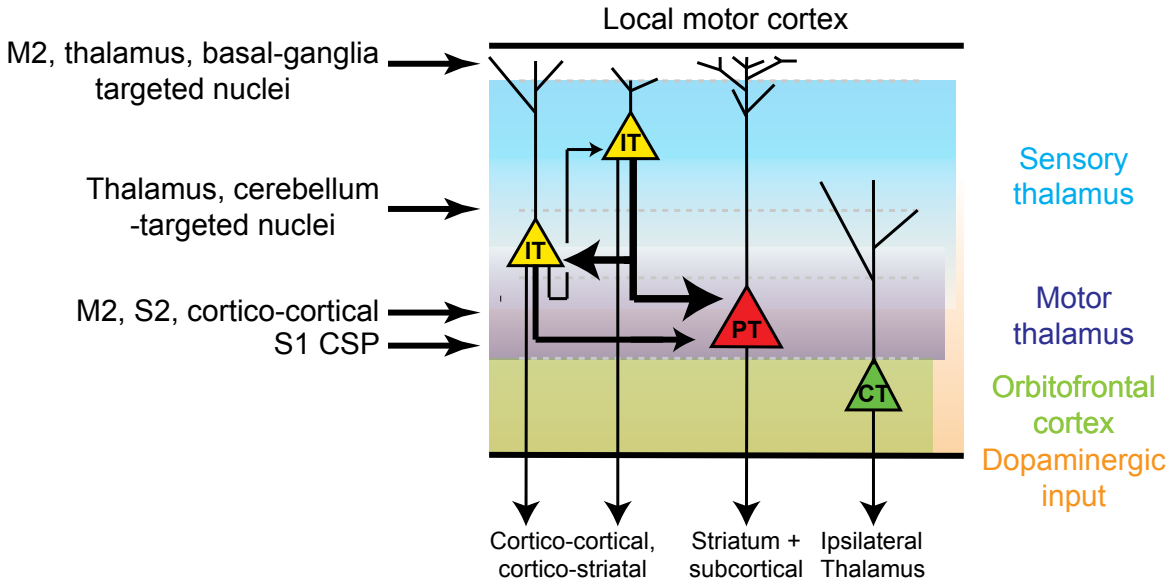

Figure 3. Long-range input to local motor cortex

Summary diagram of long-range area-specific input to local motor cortex cell types as described in the main text. The more motor-related a source area is, the higher its likelihood to directly innervate pyramidal tract (PT) neurons. Conversely, sensory source areas preferentially target cells in upper layers of motor cortex. M2, secondary motor cortex. S1 CSP, primary somatosensory cortex cortico-spinal. IT, intratelencephalic. CT, cortico-thalamic.

2011; Petrof et al., 2015).

Input from secondary somatosensory cortex (S2) innervates all layers of M1, with a bias towards superficial layers (Suter and Shepherd, 2015). Layer 5 PT neurons receive input from S2 and S2 projections to M1 can excite layer 5B neurons projecting back to S2. Retrosplenial cortex projects strongly to posterior M2 layer 5 IT-, layer 5 PT- and less so to CT neurons (Yamawaki et al., 2016). Due to its presynaptic connectivity, this projection is suggested to link cortical networks for movement execution to those implicated in spatial memory and navigation. Thalamic input innervates both, IT and layer 5 PT neurons but avoids CT neurons (Yamawaki and Shepherd, 2015). Thalamo-cortical neurons presumably receiving information from the cerebellum project mainly to the layer 3/5A border whereas those relaying information from the basal ganglia project predominantly to layer 1 (Kuramoto et al., 2009).

From these studies it seems as if the more functionally motor-related a source area is assumed to be (e.g. M2, nuclei of the thalamus receiving motor-related input from the striatum or cerebellum, S2), the more likely it is for that area to have direct, monosynaptic access to layer 5 PT or cortico-spinal projection neurons. Accordingly, the more functionally sensory a source area is (e.g. S1, posterior nuclei of the thalamus), the more likely it is to innervate upper layer 2/3 or layer 5A neurons.

Local motor cortex circuits have been shown to receive dopaminergic input predominantly in deeper layers from the substantia nigra (SN) and the ventral tegmental area (VTA). Ablation of these fibers specifically in M1 (but not prefrontal cortex (PFC) or striatum) impaired rotarod skill learning in rats, suggesting a prominent role for dopamine signaling in M1 during motor skill learning (Hosp et al., 2011; Hosp and Luft, 2013; Molina-Luna et al., 2009). Ablation of dopaminergic signaling was further found to cause aberrant synaptogenesis, inferred by two-photon imaging of motor cortical spine dynamics (L. Guo et al., 2015).

3.4.2 Long-range projections and motor loops

Motor cortex sends numerous projections to many different targets. Generally, an emerging picture is that even within a local motor cortex circuit and within the same top-level cell class multiple parallel output pathways are embedded which demonstrates substantial diversity in the projection pattern even of neurons in close spatial proximity. For example, in somatosensory cortex, pyramidal neurons in layer 2/3 send axons either to motor or secondary somatosensory areas (Yamashita et al., 2013). Similarly, deep layer 5 neurons in visual cortex have been shown to project to distinct secondary visual targets (Glickfeld et al., 2013).

3.4.2.1 Cortico-spinal tract

Classically, M1 is described as the area in which a substantial part of the neurons constituting the corticospinal tract (CST) reside. While this area is not the only source of corticospinal fibers, the M1-originating projection is the densest (macaques: Maier et al., 2002) and topographic (cats: Martin, 1996, rats: Ueta et al., 2014). Note that in primate premotor cortex (PM) there was no discernible topography and cortico-spinal innervation maps obtained by electromyography (EMG) in proximal and distal muscles overlapped completely (Boudrias et al., 2010). Throughout evolution the amount of neocortex giving rise to corticospinal projections has massively increased suggesting an equally large growth in the importance of that pathway for movement control (Barton, 2010). However, there are marked differences in the organization of the CST in humans, primates, cats and rodents.

About 90% of CST-fibers decussate at the level of the medulla, resulting in two tracts that project throughout the whole length of the spinal cord: A dorsal and a lateral tract, the former being more prominent in rodents, the latter is more prominent in primates and humans (Bareyre et al., 2005). The CST densely innervates the region of the spinal cord where motor neurons controlling distal muscles are located. Likely, species-dependent differences in contributing areas and termination zones in the spinal cord can explain the differences in the susceptibility to CST lesions and the various descending reticulospinal systems. For example, lesions to the CST in the primate greatly impaired fine digit control but other functions such as walking and climbing recovered rather quickly (Murata et al., 2008; Zaaimi et al., 2012). Subtotal lesions were far less effective, suggesting a great potential for plasticity in the spared fibers which is of major interest in clinical contexts. In rats, lesioning the CST via pyramidotomy in the medulla also leads to a reduced success in pellet handling tasks, more so than rubrospinal lesions do (Whishaw et al., 1998), which, when put in line with primate data, suggests an evolutionarily progressively increasing role of the CST in hand control. The primate reticulospinal tract originating in the brainstem also has access to motor neurons controlling hand muscles but lesions to this descending system have a much more profound effect on balance and righting than digit control (Lemon et al., 2012). Thus, from lesion studies, it has been postulated that primates rely more on cortical brain structures for fine movement generation than rodents do; for the latter, the reticulospinal system is of greater importance. In support of this notion, a study provides correlative evidence for the hypothesis that the more dexterous an animal is, the higher the density of CST innervation or direct access of the cortex to motor neurons is (Heffner and Masterton, 1983).

Likely, descending input steers movement generation through spinal interneurons. Mirror neurons discharge in the absence of any noticeable EMG activity. Thus, cortico-spinal neuron activity can be dissociated from actual muscle or, by extension, locomotor activity (Fetz and Finocchio, 1971). Additionally, delays between discharge of cortico-spinal neurons and muscle activity are long, clearly showing that the CST projection alone does not recruit spinal motorneurons above

threshold but has to act in parallel with other pathways such as the reticulospinal system. The projection of the CST is particularly dense in the intermediate layers, where spinal interneurons reside. Depending on the origin of the CST, spinal termination zones are markedly different. In mice, M1- and S2-originating CST share similar spinal trajectories. However, S2-originating fiber terminations zones were found rather in the dorsolateral horn of the spinal cord whereas M1-orignating fibers terminate more ventrally (Suter and Shepherd, 2015).

A classic example through which the CST provides a powerful influence on hand movements is the propriospinal projection originating between spinal cervical level C3 and C4, which has been shown to be critical for hand dexterity in macaques (Kinoshita et al., 2012). In line with this and a particularly strong argument for the claim that motor cortical activity has a major influence on movement generation is the fact that in some, but not all primate species the CST synapses directly on motor neurons. Contacts of cortical neurons on motor neurons have not been found in less dexterous animals such as cats (Illert et al., 1976) or rodents (Alstermark, 2004; Alstermark et al., 2004). Interestingly, it has been found that, while initially present, cortico-motorneuronal contacts onto forelimb motor neurons are greatly reduced in the rat in the course of postnatal development (Maeda et al., 2015) (though weak cortico-motoneuronal connections also exist for foot and tail motorneurons, see Jankowska et al., 1975). The purpose of direct cortico-motorneuronal connections has been speculated to help fractionating movements such that they might provide a better substrate for learning and internal models. The organization of cortico-motorneuronal layer 5B neurons resembles that of a mosaic with cell groups connected to different hand muscle motor neurons intermingled within each other and spread out over a large surface area (macaque: Rathelot and Strick, 2006). Thus, there didn't seem to be a clear, fine-grained somatotopic mapping of hand muscles within M1. Interestingly, it has been shown that cortico-motorneuronal cells in primate motor cortex were rather tuned to the way a muscle was used rather than to individual muscles themselves or synergies of multiple muscles (Griffin et al., 2015). This suggests separate populations of cortico-motorneuronal cells for different functional uses of a muscle, a concept that doesn't necessitate topographic projections.

The origin of the CST from many cortical areas (motor and premotor cortices, somatosensory cortices) make it seem unlikely that there is one single function of the CST. Rather, many different functions have been ascribed to this projection but, due to technical limitations, none of them was carried out in a cell type- or projection target-specific manner yet.

One intriguing possibility is that a major function of the CST, by virtue of its activity carrying a motor command, is to facilitate the distinction between self- and non-self-generated movement. Studies found that sensory fibers in the dorsal horn of the spinal cord can be presynaptically inhibited in a behaviorally relevant context through primary afferent depolarization both in primates (Seki et al., 2003) and mice (Fink et al., 2014). It is suggested that CST fibers originating in somatosensory cortices might provide one of the major inputs for such a suppressive mechanism

(Anderson et al., 1962). This connectivity might thus be used to cancel out proprioceptive input impinging onto the spinal cord during self-generated movement.

3.4.2.2 Cortico-cortical projections

Layer 2/3 IT neurons project mainly cortico-cortically while layer 5 IT neurons, at least collectively, also project cortico-striatally but the extent to which individual cells possess bifurcating axons (cortico-cortical and cortico-striatal) is unknown.

There is strong exchange of information between M1 and M2 on the level of layer 2/3 and layer 5 IT neurons (Ueta et al., 2014). Axons originating in M1 preferentially innervated layer 2/3 of M2 and axons originating in M2 preferentially terminated in layer 1. Based on analogies from sensory cortex, this suggests that the projection M1 --> M2 is more of the feedforward-type, whereas the M2 --> M1 projection appears to be more feed-back in nature (Ueta et al., 2014). Feed-back projections from the vibrissal motor cortex to S1 were shown to provide very heterogeneous information such as information about whisker angle and responses to whisker touch and movement (Petreanu et al., 2012).

Callosal projections from the other hemisphere were suggested to be latent (not tonically active) but could possibly contribute to motor control under conditions such as recovery after unilateral injury (Brus-Ramer et al., 2009; Li et al., 2015b).

3.4.2.3 Cortico-striatal projections

The basal ganglia are frequently implicated in gating of actions and locomotion and described as being involved in reinforcement learning. Most of cortex including sensory, motor and association areas project to the striatum, in rodents, cats, primates and humans. Generally, the input is bilateral but with clear ipsilateral dominance. Moreover, motor cortex is monosynaptically exciting striatal projection neurons but communication from the striatum to motor cortex involves at least two synapses via the thalamus. It is also worth to note that, unlike motor cortex, the basal ganglia do not have direct access to the spinal cord. Instead, their unique position in the motor control hierarchy also stems from the fact that ipsilateral striatum is the only subcortical structure that is innervated by both principal projection neurons of the motor cortex, layer 5 IT and layer 5 PT neurons. The basal ganglia have been implicated in numerous functions, including action selection, motor control, sequence learning and habit formation. In mice, it has recently been found that movement velocity depends bidirectionally on the activity of direct and indirect pathway medium spiny neurons (MSNs) (Kravitz et al., 2010; Yttri and Dudman, 2016). It is conceivable that, by modifying the cortical excitatory input to the striatum, locomotion speed

of animals can be directly manipulated. To what extent projection-specific input can account for each of these functions is unclear.

Layer 5 IT neurons have been shown to project to both direct and indirect dorsolateral striatal spiny projection neurons with about equal functional strength (Kress et al., 2013) despite an anatomical study which capitalized on monosynaptically restricted rabies virus tracing which found a bias for motor cortical projections onto indirect pathway MSNs (Wall et al., 2013). In the behaving monkey, the activity of IT-type cortico-striatal projection neurons has been found to be highly directional with respect to the reach movement and displaying sensory responses but lacking a correlation with muscle load (Turner and DeLong, 2000). This led to the conclusion that information relayed to the striatum is distinct from that transmitted to the brainstem via layer 5 PT neurons (Note however, that individual layer 5 PT neurons are capable of innervating both striatum and brainstem targets (Kita and Kita, 2012)). In a primate model of Parkinson's Disease (PD), it was recently found that after induction of Parkinsonian symptoms, PT-type neurons activity was generally reduced and the temporal structure of the activity was abnormal (Pasquereau et al., 2016). Interestingly, cortico-striatal IT neurons seemed to be much less affected, suggesting that a motor cortical source of PD can likely be attributed to layer 5 PT neurons. This further supports the notion that striatum-targeting IT and PT neurons convey a very different type of motor-related information.

Layer 5 PT neurons, like IT neurons, also project to both direct and indirect dorsolateral striatal spiny projection neurons albeit about twice as strong to direct pathway neurons (Kress et al., 2013). Layer 5 PT branches at striatal levels could provide a "go"-signal to the direct pathway in the striatum which might serve to disinhibit movement centers in the midbrain / brainstem. That could add to the excitatory drive arising from layer 5 PT neurons. However, this simple view is complicated by the fact that the distinction between direct pathway, the activity of which being assumed to be movement promoting, and indirect pathway, the activity of which being assumed to be movement repressing, has been challenged. Cui et al. found in mice that during locomotion, both pathways become activated albeit differentially depending on the direction (but not vigor) of the subsequent movement.

3.4.2.4 Cerebellar loops

Cerebellar research has a long-standing history for its implication in motor control. Most of the functional implications, however, have been derived from studies examining the consequences of damage to the cerebellum. By raw neuron numbers, the cerebellum is suspected to be empowered with an impressive information processing capability (Zagon et al., 1977). The cerebellum receives direct proprioceptive input through the spino-cerebellar tract and is target of motor cortical input by means of the pontine nucleus (Coffman et al., 2011; Schmahmann et

al., 2004; Ugolini and Kuypers, 1986) which might both provide a mixture of sensory feedback and efference copy signals that is useful in state estimation according to the optimal feedback control theory (Todorov and Jordan, 2002). In this framework, parallel fibers made by granule cells could provide an error signal to Purkinje cells (Garwicz, 2002; Kitazawa et al., 1998) which could update internal models, storage of which is a function that has often been attributed to the cerebellum (Haruno and Wolpert, 1999; Imamizu et al., 2000; Liu et al., 2003; Miall and Wolpert, 1996). In line with this, it was found that splitbelt walking was impaired for predictive but not reactive locomotion in cerebellar humans (Morton, 2006). Cerebellar output is routed either through reticulospinal centers or, resembling more a feedback projection, information from the cerebellum is sent to motor cortex via the thalamus (Dum, 2002; Holdefer et al., 2000) providing a way by which the cerebellum might influence motor command processing.

Spinal proprioceptive feedback might be stored in an experience-dependent manner in internal models in cerebellar circuits and applied to influence subsequent movements. Experimentally, it is hard to distinguish between the role of direct movement commands and prediction derived from their respective internal models. However, one can assess if the cerebellum provides predictions to the brain. This has been tested in humans in a reaching task in which part of the reach was performed under visual guidance and upon a cue, the visual feedback was removed and subjects were instructed to continue reaching to the target. If the cerebellum merely represents the current position of the hand as estimated by sensory feedback, it should inevitably result in misses by an offset equal to the reaching velocity times sensory delay. That was not the case: people maintained target accuracy quite well suggesting a prospective coding of motor commands. The researchers then perturbed cerebellar activity by transcranial magnetic stimulation (TMS). If the cerebellum was site of the prediction, the subjects should make displaced reaches which is what the researchers observed (Miall et al., 2007). Inactivation of the dentate nucleus in primates resulted in monkeys unable to retrieve overlearned motor sequences but they could still learn new ones (Lu et al., 1998). This effect was specific to the hemisphere ipsilateral to the hand used for the execution of the movement sequence. Thus, control of unfamiliar movements might not depend on the cerebellar motor loop but, after learning, effector-specific internal models which have been created and refined in cerebellar circuitry are crucial for efficient movement execution.

3.4.3 Ethological movement topography within motor cortex

Topography in visual cortex can differ dramatically across species. For example, while cat and primate visual cortex shows a functionally organized, periodic arrangement into orientation columns, this is not the case in rodents, possibly owing to the mere size of their visual cortex (Ohki et al., 2006, 2005; Vanduffel et al., 2002). Interestingly, this difference does not hold up

for motor areas. Instead, in motor areas a seemingly full array of behaviors is mapped onto the cortical surface limited only by the biomechanics of the body (Harrison et al., 2012; Hira et al., 2015) while neurons capable of eliciting electromyographic activity were intermingled for different muscles (Dum and Strick, 2005; Griffin et al., 2015). It was hypothesized that the motor cortex surface is subdivided into behaviorally – or ethologically – relevant regions (Brecht et al., 2004; Graziano, 2016; Graziano et al., 2002). In spite of potential technical artefacts due to electrical stimulation it remains to be determined what the underlying computations generating such stereotype behavior are.

Evidence that motor maps can comprise functional units in motor cortex comes from learning studies: Skill training resulted, in motor cortex, in reorganization of movement representations (Kleim et al., 1998; Molina-Luna et al., 2008; Pruitt et al., 2016) which is accompanied by synaptogenesis (Kleim et al., 2002). The expansion of forelimb maps as measured by ICMS was transient but specific to the trained limb and, after further training, returned to pre-training levels which suggests a normalization of maps after consolidation of the respective skill. Accordingly, map size did not correlate well with motor performance in the late phase of learning.

Thus, motor maps, while far from understood, are an observable feature of motor cortex organization with the capability to undergo plasticity during learning.

3.5 Sensorimotor learning

Motor cortex has long been postulated to be a prime site for the generation of movement commands (Drew and Marigold, 2015). The connectivity with many brain centers involved in locomotion clearly provides support for this notion. Still, the exact cortical contribution to acute movement generation has been subject of intense debate as well as the extent to which motor cortical circuits are implicated in learning of novel motor skills. A significant roadblock is the poor understanding of cognitive processes that drive activity which ultimately results in movement. Thus, despite greatly improved recording techniques, the interpretation of the relationship between recorded activity and measured behavior is open to debate (Schwartz, 2016). This section will focus on what is known of how, first, motor cortex is involved in learning of skills and second, how execution of newly learned skills might be under motor cortical control.

3.5.1 Behavioral adaptation in sensorimotor learning

On the behavioral level, motor skill learning is usually characterized by shortening of response time and performance of faster, more stereotypical and thus less variable movements which could be ascribed to an increase in efficiency of information processing (Cohen and Nicolelis, 2004). Owing to the obviously much greater behavioral repertoire, the exact trajectory of motor skill learning might be different in humans. In humans, improvements in performance largely consisted of a reduction in trial-to-trial variability and increase in movement smoothness (Shmuelof et al., 2012).

Error-based learning is the driving force behind many sensorimotor adaptations which occurs on a single trial basis (Diedrichsen, 2005; Thoroughman and Shadmehr, 2000; Tseng et al., 2007). Apart from the cerebral cortex, the cerebellum also seems to play an important role in learning based on errors from the sensory periphery (Tseng et al., 2007). This form of learning exploits a signed error signal in order to infer in which direction to modify motor behavior. While error-based learning can reduce the average error to zero, it does not provide a means by which to improve performance beyond mere error correction. For example, professional musicians not only depend on being able to perfectly execute a given finger movement but performance must also be reproducible, that is, excessive variability over trials needs to be reduced.

3.5.2 Temporal difference learning as a model for reinforcement learning

Reinforcement signals provide a measure on success or failure of movement execution

(Wolpert et al., 2011). Since these signals are unsigned in nature and therefore do not inform about the directionality of the behavioral change, the motor system has to explore various possibilities by trial and error. Thus, this form of learning tends to be slow, which becomes especially apparent if the action is complex (e.g. a long sequence) and the collection of the respective reward is temporally discontinuous.

A mechanistic hypothesis how reinforcement learning could be explained was put forward by the classic Rescorla-Wagner model which used the reward (omitted or provided) as a teaching signal in each trial to associate a stimulus with the outcome (Rescorla and Wagner, 1972). This model, however, could not explain behavior in the case of discontinuous stimulus-reward pairings. Moreover, if a trial (ultimately an arbitrary division of time) was not finished, there could never have been learning because the reinforcement signal was missing. Temporal difference (TD) methods were originally invoked as a means to optimize computation during supervised learning (Sutton, 1988). As Sutton already noted early on, this approach could also be applied to account for animal behavior in learning experiments. TD tries to predict, at any given time, the cumulative future reward. It was soon observed that the phasic firing of midbrain dopamine neurons in the VTA closely resembled the error term in the model (coined reward prediction error, Hollerman and Schultz, 1998) which suggests the brain actually employs algorithms similar to TD as learning mechanisms.

TD learning computes predictive signals but does not select optimal actions (Suri, 2002). On the other hand, numerous studies demonstrate that animals are capable of incorporating reinforcement cues into optimizing their behavior. This raises the question how optimal action selection is performed in a neuronal network. The midbrain VTA is origin of a widespread dopaminergic projection with targets throughout the brain and prominent innervation in striatum and motor cortex structures implicated in motor skill learning (Hosp et al., 2011; Molina-Luna et al., 2009). It is conceivable that the location at which dopaminergic input and motor executive signals coincide are those at which actions are selected based on their salience to the acting animal. The pairing (or absence of pairing) of pre- and postsynaptic activity with dopaminergic input can then modify synaptic efficacy (Reynolds et al., 2001).

3.5.3 Learning-related structural plasticity

General learning theories suggest, as a manifestation of learning, either changed activity patterns that are somehow stored in neuronal networks or structural plasticity such as changes in protein synthesis leading ultimately to changes in morphology that manifest themselves in addition or removal of dendritic spines, the most common site of excitatory synapses. Much work has been dedicated to the understanding of the processes and mechanisms involved in learning of skilled movements and, as a consequence, a vast array of learning-related structural plasticity

and activity dynamics has been reported, few of which stratified the investigated cell types by axonal targets. Since the early days of neuroscience research it was thought that structural changes underlie long-term information storage such as memory formation. With the advent of high-resolution imaging technology such as two-photon imaging, direct observation and testing of these hypotheses became possible.

Using slice electrophysiology, early studies have shown that upon motor skill learning the efficacy of local motor cortical synapses is increased by long-term potentiation (LTP) (Rioult-Pedotti et al., 2000). The question whether individual synapses can be the site of learning was resolved subsequently by using two-photon spine imaging of layer 5 pyramidal neurons. It was shown that motor skill training preferentially stabilizes new spines induced during learning and that this process is specific for different motor skills (Kleim et al., 2002; Xu et al., 2009; Yang et al., 2009) demonstrating that first, spines and, by extension, synapses are the site of learning-related plasticity and second, that different motor memories are stored in different synaptic ensembles (Hayashi-Takagi et al., 2015). Furthermore, during learning, clusters of new spines tend to form (Fu et al., 2012). Similar results have been obtained in layer 2/3 suggesting an overall similar mechanism of learning-related structural plasticity for layer 2/3 and layer 5 neurons (Ma et al., 2016). Using histological reconstructions a study found projection-specific structural plasticity in retrogradely labelled cortico-spinal neurons in the rat motor cortex during training of a forelimb-grasping task suggesting that structural plasticity depends on task demand and cell type and that cortical resources are channeled selectively to neurons important for the success of the task (Wang et al., 2011). Of note, synaptogenesis appeared only in the late phase of motor learning which might reflect the consolidation of skills (Kleim et al., 2002). It also suggests that in the early phase other structures than motor cortex are undergoing structural plasticity. Motor learning can quantitatively increase the number of axons impinging onto M1 indicating that beyond the synaptic and therefore local circuit level, dramatic morphological changes can occur in the course of acquiring a new motor skill (Sampaio-Baptista et al., 2013) .

It seems as if structural plasticity is the means by which memories are stored on a long-term time scale. The study by Hayashi-Takagi et al. has elegantly shown that newly added spines are the site of motor memory storage but it precludes the question of what determines this process. How is it decided a given spine becomes stabilized? What part of the information of a motor memory is stored in any one such synapse? What are the precise reasons underlying the structural dynamism that is frequently observed for cortical neurons?

3.5.4 Learning-related activity dynamics

It is reasonable to assume that activity of individual neurons and networks of neurons underlies the induction of structural plasticity. In theories of motor learning it is often assumed

that learning happens against a backdrop of stable neuronal representations. A study in primates found that representations of individual neurons are remarkably unstable, possibly switching between states that are behaviorally equivalent (Cohen and Nicolelis, 2004; Rokni et al., 2007). Thus, it might be, as the task becomes more difficult, the representation of task parameters stabilizes because the number of possible states that are behaviorally successful and equivalent decreases.

In mice, activity in layer 2/3 cells correlated with behavioral parameters such as whisking and licking and potentially reflected representations of task parameters (Huber et al., 2012; Komiyama et al., 2010). The representations were intermingled, consistent with the general idea that dedicated layer 2/3 subnetworks project specifically, according to their function, to deeper layers. The population level representation of individual task parameters stabilized, while activity of individual neurons could be variable. In agreement with and in extension of this finding, layer 2/3 representations maintain about an equal information content in the course of learning a lever-pull task while the same measure in layer 5A neurons steadily increased (Masamizu et al., 2014). Thus, it appears as if the activity of deep layer neurons is more likely to be shaped by learning and by gradual and directed behavioral changes that usually go hand in hand with increased task performance. Behavioral pattern formation in motor cortex might operate like a unsupervised neural network. By training, it learns how to combine inputs in order to generate purposeful output.

Cortical plasticity was also suggested to be useful for identifying the minimum number of neurons required to accomplish a task (Reed et al., 2011). In line with this idea a study found that associative learning in somatosensory cortex enhanced sparse coding but decreased total network activity (Gdalyahu et al., 2012). Thus, by learning, high fidelity neurons emerge which disproportionately contribute to the network activity.

Hebbian mechanisms might be the driving factors for inducing learning-related changes. A cellular basis for this hypothesis was provided by showing in barrel cortex that coincidental long-range input from motor cortex and intracolumnar feedforward input onto layer 5 apical tufts can induce long-lasting plateau potentials (Xu et al., 2012). A similar mechanism could be at play in motor cortex where task-relevant sensory input which is concurrently active with motor signals could enable Hebbian plasticity.

Projections from thalamus to motor cortex strengthen preferentially onto neurons which control distal, not proximal, muscles (C8 vs C4) after grasp training (Biane et al., 2016) indicating that thalamocortical synapses can also be refined in a use-dependent manner.

3.6 Sensorimotor control

3.6.1 Sensorimotor transformation

Goal-directed behavior makes use of a constant stream of sensory information that is derived from peripheral sensory organs and routed to cortical regions. In order to be able to execute an appropriate motor command, it is commonly assumed that this sensory information has to be transformed from the original sensory frame of reference to a frame of reference which is able to communicate with spinal structures and, ultimately, with motorneurons controlling muscles. There is consensus that cortex should be the site for the computation of sensorimotor transformation but how and where exactly it takes place is controversial. Evidence suggests there could be multiple sites for sensorimotor transformations.

For example, in primates M1 has been implicated in such computations since a long time (Salinas and Romo, 1998; Shen and Alexander, 1997a; Zhang et al., 1997). In cats it was found that acceleration information likely originating from group I proprioceptive afferents is used to generate motor commands during postural balancing (Lockhart and Ting, 2007). Since postural control does not require cerebral cortex, the authors speculate that the brainstem might be a critical site for this type of sensorimotor transformation. In mice, primary visual cortex (V1), posterior parietal cortex (PPC) and M1 were implicated in sensorimotor transformation (Buneo et al., 2002; Goard et al., 2016; Harvey et al., 2012)

The circuits and computations mediating sensorimotor transformations are only beginning to be understood. For it to take place in local motor cortex circuits there need to be sensory input. Evidence suggests a stratification of long-range input to motor cortex according to the functional nature of its source: Input from sensory cortices is biased towards layer 2/3 and upper layer 5 neurons and the more motor-related a source area is the higher the likelihood for it to have monosynaptic access to layer 5 PT neurons (Hooks et al., 2013; Mao et al., 2011; Suter and Shepherd, 2015).

It has been speculated on the mechanism by which sensorimotor transformations might be realized. For example, task-level variables can rarely be encoded by any one sensory signal or modality; rather, they must be estimated from many sensory modalities, requiring substantial computational power. Furthermore, sensorimotor transformations are often non-linear. Thus, apart from the sensory input layer and the motor output layer it is reasonable to assume that there is an intermediate step to recode sensory information before it can be transformed into motor commands (Pouget and Snyder, 2000).

3.6.2 Motor cortical control of movement

Pioneering studies in cats and primates have found strong correlations in the discharge pattern of motor cortical neurons and the movement the animals were to execute (Beloozerova et al., 2003a, 2003b; Churchland et al., 2012; Churchland and Shenoy, 2007; Drew et al., 2002; Griffin et al., 2015; Guo et al., 2015; Kaufman et al., 2013; Martin and Ghez, 1993; Pasquereau et al., 2016; Quallo et al., 2012; Townsend et al., 2006; Yakovenko and Drew, 2015). This has led to the postulation of motor primitives, muscle synergies or representations thereof which are active in a behaviorally relevant manner being driven by coordinated motor cortex activity (Lemay and Grill, 2004; Mussa-Ivaldi et al., 1994; Thoroughman and Shadmehr, 2000).

The conclusion that motor cortex is essential for execution of the investigated movements is seemingly at odds with a number of lesion or perturbation studies which ablated either the CST or corresponding motor cortical areas and animals were still able to execute basic motor behavior. This raises the intensely debated question what essential computation is taking place within local motor cortex circuitry. For instance, in rats, complete bilateral motor cortex lesions did not impair execution of a previously learned, reinforced and presumably non-dexterous timed lever press task, though learning of the same task was profoundly impaired (Kawai et al., 2015). It is conceivable that compensatory homeostatic plasticity took place which would have enabled other motor centers such as striatum or midbrain to perform the required movement. It could be that projections of cortico-striatal IT and layer 5 PT neurons are specifically needed to convey learning-related information which after reaching the expert stage is not essential anymore. Consistent with this it was found in humans that M1 function is necessary to consolidate motor memories (Muellbacher et al., 2002).

A follow-up study, using muscimol to acutely inhibit or optogenetics to generically disrupt motor cortical activity has found much more severe impairments during acute perturbations of activity than previously observed using chronic lesions (Otchy et al., 2015). A study in which mice learned to reach for a pellet showed that previously learned movements are susceptible to motor cortex silencing which eventually caused behavioral arrest (Guo et al., 2015). Thus, it seems as if when chronically deprived of motor cortical input, spontaneous recovery of activity patterns in subcortical structures is able to mimic cortex functions in order to sustain behavior important for survival. This is prevented by acutely perturbing activity, causing a much more severe phenotype.

Primary motor cortex is activated bilaterally during unilateral movements (Cisek et al., 2003). Insight into lateralized motor control is likely especially important in recovery of movement control in clinical conditions of unilateral injury to the CST. A recent study provided mechanistic insight into the nature of bihemispheric motor control (Li et al., 2015b). In mice subjected to a delayed forced two-choice task, acute optogenetic silencing of either motor cortical hemisphere during the late delay phase did only moderately impair task performance. However, bilateral silencing reduced behavioral performance to chance levels suggesting that activity in the

unaffected hemisphere is instrumental to restoring neuronal activity dynamics in the transiently silenced hemisphere. In line with this Brus-Ramer et al. suggested latent connections between motor cortical hemispheres which could contribute to recovery after injury.

Preparatory activity was implicated as a subthreshold form of activity driving muscles and later hypothesized to be the initial state upon which the motor cortical system efficiently produces movement (Churchland et al., 2010, 2006; Tanji and Evarts, 1976). Accordingly, a study in monkeys using an instructed delay reaching task found that preparatory activity remained within an output-null dimension prior to execution of the movement. Upon an as yet unknown signal, this dynamics then shifts into an output-potent state which enables movement (Kaufman et al., 2014). A large part of pre-movement activity was found to be indicative of movement timing rather than movement type suggesting different neuronal mechanisms for what is supposed to be done as opposed to when it should be done (Kaufman et al., 2016). Thus, preparatory activity itself does not necessarily lead to movement output but enables movement control signals that do.

The role of motor cortex might be in generating patterns of activity useful for movement execution (Churchland et al., 2012). Patterns resembled rotational oscillations in low-dimensional neuronal state space. Such oscillations are useful basis functions to generate more complex patterns. Supporting evidence came from EMG recordings which were well approximated by a linear combination of the previously recorded motor cortical patterns.

Decision-making can be seen as a subthreshold activation of the very same neurons that execute the action. The ramp-to-threshold model proposes an action is executed once the activation of a given neuron (or ensemble) exceeds a certain threshold. In this respect, decisions are intimately linked with action preparation and preparatory activity might at least in part also reflect such a process. Such activity patterns have been observed in primates (Maimon and Assad, 2006) and rodents (Murakami et al., 2014).

3.6.3 Predictive motor control

The first level of sensory feedback processing happens within the spinal cord. In many species, the spinal cord can support control of a rich pattern of behaviors such as scratching, wiping and basic locomotor patterns. Traditionally, reflexive movements are viewed as a prime example for feedback control where the output of the system modifies its input. Consider the example of a stretch receptor in a muscle: If the muscle is stretched, it becomes active and excites the muscle which contains it which causes contraction and thus shortening of the muscle, counteracting the initial stretch (Poppele and Terzuolo, 1967; Roberts et al., 1970). Even in this rather simple example, the sensitivity of the stretch receptors can be adjusted by gamma motor

neurons that are themselves controlled by supraspinal centers. Clearly, pure feedback control is neither exclusively implemented nor is it sufficient to explain the range of capabilities of the motor system. For example, a stunning feature of the motor system is its ability to carry out very fast and accurate movements. In particular, some in-flight corrective movements are carried out faster than the inevitable delay between peripheral sensors (e.g. proprioceptors) and central structures. Since those movements still maintain target accuracy there need to be mechanisms that allow for prospective, feed-forward motor control, a field termed predictive coding. A core concept in predictive coding (Rao and Ballard, 1999) is the internal model, an experience-dependent approximation of the external world, which is employed to estimate sensory consequences of motor actions (Jordan and Rumelhart, 1992; Miall and Wolpert, 1996). In predictive coding, descending, top-down projections convey predictions. One application is to distinguish self- from environmentally-generated sensory feedback. Additionally, by computing the difference between actual and expected sensory outcome of an action, the nervous system could derive an estimate for the error in movement execution which is of immense value for motor learning. A likely candidate structure for such a computation is the cerebellum (Miall et al., 1993; Pasalar et al., 2006).

For internal models to be effective, learning is necessary to adjust the model to changing environments. For example, throughout life the relationship of force necessary to move a limb is a function of body weight. On a shorter timescale, muscles fatigue needs to be compensated for in order to maintain movement precision. A third scenario could be inaccurate predictions because of uncertain beliefs which result in noisy internal models. The brain has to cope with these situations and how it does so has been topic of motor control research. In experiments, humans were asked to perform reaching movements to targets placed equidistantly on a circle around the starting point. This was easily accomplished. When different force fields were applied to the manipulandum, movements were characteristically distorted but after repetition, normal movements were produced which matched the force-field free control condition (Shadmehr and Mussa-Ivaldi, 1994). This is taken as an example in which the internal model of the reaching movement was updated according to the task's environment. Thus, repetition and learning plays a critical role in motor adaptation. Internal models and efference copies should also be specific to the nature of movement command being transmitted. For example, during auditory- and visually-guided movements, even if they are qualitatively similar, somehow it needs to be distinguished to which areas to route the efference copy to. Perhaps the coincident arrival of corollary discharges with neuromodulatory signals could solve this problem.

Volitional movements are goal-driven and the ability to successfully execute a movement depends on good predictions about what motor output will be necessary to achieve the movement goal. This implies a temporal component. For example, movement speed cannot be inferred by measuring only one time point. Rather, it is an integral of the distance covered over time. Thus, the brain needs to take into account the history of movement in order to generate an estimate of

velocity which can then be used to predict future situations.

The flexibility and adaptability of internal models stored in motor cortex can be capitalized on in clinical conditions of nervous system injury. Not only do brain-machine interface (BMI) rely on motor cortex activity to inform patterns of spinal cord electrical stimulations and re-enable simplest motor control in patients with spinal cord injury (Shanechi et al., 2012) but the technology can also be used to study the learning of new internal models in human patients (Golub et al., 2015).

3.6.3.1 Optimal control

The human body has more degrees of freedom for motor performance than needed to perform any particular task within the range of its biomechanical properties. While this is desirable to create flexible motor behavior, one of the puzzling questions that motor neuroscience is dealing with since more than 80 years is: how does neuronal activity generate purposeful, coordinated movements in the face of the extremely complex peripheral motor system (e.g. limbs, joints and muscles)? By virtue of the fact that despite these obstacles, movements are in essence smooth, it was hypothesized that sensory feedback plays a prominent role in guiding movements (Scott, 2004). However, the relatively large sensory delay (e.g. proprioception, at least 50 ms) is at odds with seemingly smooth movement profiles and fast corrective behavior. Therefore, an optimal controller was postulated which, moment-by-moment, evaluates on the basis of an optimal feedback law the state of the motor system predicted by internal models and what change in movement details would be necessary for optimal task performance (Todorov and Jordan, 2002). Optimal task performance is often equaled to following a desired trajectory of movement. However, because of the many degrees of freedom the skeleton allows, there will almost always be more than one optimal solution. This is commonly referred to as the inverse problem: Finding and computing a motor command which produces an optimal movement trajectory in the face of many possible options. In optimal control, the inverse model which provides the mapping of motor commands to movement is thought to be realized in motor cortex. In this framework it follows that motor cortex issues fully-processed motor commands which is in sharp contrast to the active inference framework (see next section)

Supporting observations for optimal control come from human psychophysics in which participants were asked to execute a reaching movement while the trajectory was perturbed in-flight. If there was a pre-defined trajectory for the reaching movement there should be a great impact on movement success. However, almost effortlessly humans can correct for these perturbations. This is taken as evidence that humans perform sensory feedback guided movements rather than planning a complete movement before actually executing it.

A recent study showed that movements can be decomposed into parts in which accuracy

requirements of movement execution are low and chunks in which accuracy requirements are high (e.g. approaching a point target). By taking into account accuracy demands only later in the movement (a feedback type of information), the initial reaction time could be measurably reduced (Orban de Xivry et al., 2016). This implies that movement goals are updated during the execution of the movement. In cases of multiple, potentially competing, motor plans initially the average of the control policies is selected and the appropriate motor plan for task success is selected later on (Gallivan et al., 2016b).

3.6.3.2 Active inference

The brain operates on predictions of what it expects to sense and incorporates that into sensory processing at the earliest stages. Consider this example: The cornea provides the majority of refraction to focus the light on the fovea. A mild abrasion in one eye as it can happen by unintentionally dipping a finger into your eye results in an interesting phenotype: Vision with both eyes open will not allow you to see crisp images of the environment. If you close the affected eye you can see crisp images (albeit without stereovision). Clearly, there is fundamental processing by the brain involved to merge the two images from both eyes' retinas and additionally, we cannot voluntarily ignore one eyes' sensory input. This illustrates that perception – the making sense of raw sensory input - is an inference process and not a mere representation of the world (Helmholtz, 1860). By employing a generative model which creates predictions on perception, the brain can infer causes of sensory impressions. We learn to estimate what could be the reason for a change in what we sense. However, often many causes can lead to the same sensory consequence for example in order to execute a reach many muscles can be used to achieve the same movement goal. A solution to this problem is using multiple internal models (predictions) which inform about how causes interact with each other. But that again requires prior knowledge in order to infer how the interaction looks like and how it changes in response to a change in environmental input. One major task for cortex might be to constantly finesse these prior beliefs in order to generate an accurate as possible prediction. In sensory cortex, hierarchical processing can be used to approximate the causes of sensory input and then minimize the difference between the predicted and actual input. Predictions are therefore created in higher cortical areas and passed down the hierarchy by descending projections. (Processed) Sensory information is relayed via ascending projections which in active inference is termed collectively prediction errors. The aim of the entire computation and, by extension, behavior, is to minimize prediction error (Friston, 2010). This can be achieved by either adjusting the prediction (cortical computation) or changing the sensory input such that they are in alignment with the prediction (Adams et al., 2013). The latter case can be realized by active behavior. From an active inference point of view, behavior serves the purpose and is the action required to minimize prediction errors (Friston, 2010).

In contrast to optimal control, where descending motor cortical activity via the CST is

assumed to be motor commands, active inference posits that predictions are sent and then compared with prediction errors from the sensory periphery to generate motor output. In active inference, the inverse problem is thus solved on the level of spinal circuitry. This difference becomes important when addressing the question of motor control experimentally: Motor commands – activity directly capable of driving muscles – is context-invariant and should only depend on the current state of the muscle. However, predictions should depend on the context in which they are made, for example depend on the history of past uses or statistics of the environment such as for example how likely it is to encounter a certain perturbation. An example for an ascensing projection conveying the proposed prediction error could be Clarke's column in the spinal cord, whose neurons might convey ascending proprioceptive prediction error to the cerebellum, potentially also via the lateral reticular nucleus (LRN) (Hantman and Jessell, 2010; Pivetta et al., 2014).

4. QUESTION ADDRESSED

From electrical microstimulation studies, one can assume motor cortex is essential for or at least involved in movement generation but data from lesion studies has been equivocal to answering this question. Moreover, motor cortex in rodents is a surprisingly understudied area of the brain with respect to its *in vivo* functions and behavioral relevance of different cell classes. As outlined in the introduction, functional cell classes tend to align with their connectivity but data on activity patterns of defined projection cell classes has only recently begun to be collected.

I hypothesized that ongoing motor cortex activity is instrumental during sensory guided motor behavior and designed a task in which I could compare spontaneous and hence expected mouse behavior, to reactive behavior in response to an unexpected, unpredictable sensory stimulus. Using head-fixed mouse behavior, I gained control over the visual scenery mice experienced and during awake mouse behavior, either manipulated motor cortical activity by optogenetic methods or chronically recorded activity from genetically defined projection cell types by two-photon calcium imaging in the course of learning.

In particular, I raised the following questions:

1) Under what conditions is (ongoing) motor cortical activity critical to successful, goal-directed behavior? Does forced visually-guided behavior require motor cortex?

2) Does activation in local motor cortex change as a result of learning? As mice learn the task, execution of behavior becomes more efficient. Could changes in neuronal activation underlie the increased efficiency?

3) Does activation in cell types change differentially? From knowledge of connectivity, superficial layer 2/3 neurons could be assumed the input stage of local motor cortex circuits and activity of layer 5 PT neurons might mediate the motor output. These different functions raise the possibility that activation changes cell type-specificly.

4) How is mouse behavior reflected in neuronal activity patterns? Activation in motor cortex might not only be plastic during long-term learning-related changes but might also be informative of mouse behavior in individual trials.

5. RESULTS

5.1 Task design and mouse behavior

In order to study the activity of motor cortex during volitional movement control I modified a setup (**Figure 4A**) which had previously been used for two-photon calcium imaging during awake mouse behavior (Dombeck et al., 2009; Leinweber et al., 2014). Mice were head-fixed but otherwise free to run on an air-supported spherical styrofoam treadmill. Movement of the styrofoam ball was translated into visual feedback by custom-written software in LabVIEW which was projected onto a toroidal screen positioned in front of the mice covering the majority of their visual field. Mice were water-restricted to increase incentive to perform the task. The task required mice to navigate a virtual tunnel towards a distant target region marked by a blue cylinder upon the arrival at which a water reward was delivered and, after a 5 s time out period in which a grey screen was shown, a new trial was initiated by displaying the tunnel in the same orientation mice finished the traversal (**Figure 4B, see Movie_ExpertTraversal**). The width of the virtual tunnel was held constant but increased in length with increasing expertise such that mice obtained approximately 1 - 2 rewards / min. The final length of the virtual tunnel was adjusted such that mice, when running in a straight line, had to traverse approximately 6 m of physical distance which took on average 28 ± 2 s (n = 28 mice, ± SEM) to accomplish. The virtual tunnel was textured with either a

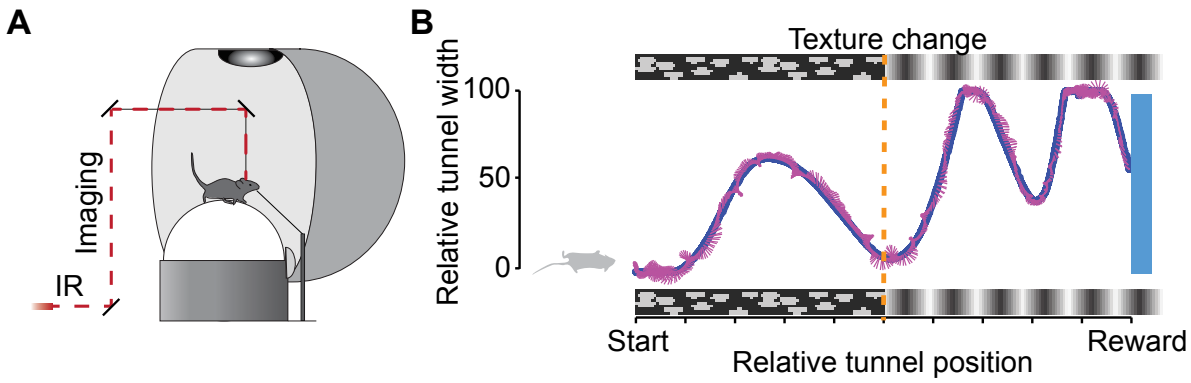

Figure 4. Task design and mouse behavior

(**A**) Schematic of the setup. Water restricted mice were head-fixed and trained to navigate a virtual reality environment by means of visual feedback generated by self-motion on an air-supported spherical styrofoam treadmill. During learning, neuronal activity was recorded by two-photon calcium imaging. (**B**) Top-down view of the virtual tunnel and sample trajectory (blue line) of an individual traversal through the tunnel. Angular velocity vectors in magenta. The tunnel walls were textured with either spherical or grating patterns, referred to as texture A or B, respectively, in subsequent sections of the main text, where applicable. Textures changed at the midpoint of the tunnel. Blue cylinder marks reward zone.v

pattern of grey spheres on black background ("spheres") in the first half of the tunnel or sinusoidal gratings ("gratings") in the second half of the tunnel. Care was taken patterns always changed at exactly half-way through the tunnel except for specific recordings in layer 5 IT neurons where a uniform texture pattern was displayed in a subset of trials (see Experimental Procedures). The texturing of the tunnel walls was meant to provide an additional cue for navigation and to confer directionality of the tunnel such that mice, when navigating from spheres to gratings, could infer they were running in the direction of the reward whereas when running from gratings to spheres it would imply running towards the dead-end part of the tunnel. The navigation task was designed such that either two-photon imaging could be carried out concurrently while the mice were actively behaving in which case an imaging objective was positioned over a cranial window to record neuronal activity (**Figure 4A**, see Experimental Procedures). Or, on a different setup, targeted optogenetic activity manipulation could be achieved to directly assess the importance of motor cortical areas during active behavior (**Figure 6A**). Experiments were performed daily and a session typically lasted 45 min in which mice navigated in the virtual environment ("VR"). When imaging there was an additional 10 min of recording performed while mice actively behaved under identical conditions as the rest of the session but the projector was switched off ("DARK").

Concurrent to either activity recording (imaging) or manipulation (optogenetics) I measured many parameters related to mouse behavior such as forward or turning velocity, position and heading in the virtual environment or, by processing of video recordings, licking behavior. Much spontaneous turning with few targeted runs was initially characteristic of mouse behavior but, with increasing experience, behavior became progressively more goal-directed. Of note, mice were free to initiate movement at will and did so spontaneously (**Figure 5A**). Relative to the position in the virtual tunnel, I quantified different behavioral epochs. Spontaneous turning events happened with about equal probability throughout the tunnel with a slight bias towards the ends. This was likely due to superstitious mouse behavior at the ends of the tunnel. Running onsets in both VR and DARK conditions mostly occur at the beginning of the tunnel. This is expected since mice were required to continuously run in order to obtain water rewards but at the time when the water droplet is dispensed mice usually slow down or stop to lick. Accordingly, licking is equally biased towards the ends of the tunnel. Of note, mice start to lick in an anticipatory manner before reaching the target location indicating that the association between blue cylinder and water reward has been successfully formed. To further explore this anticipatory licking, the licking events were split by sessions and their likelihood of occurrence plotted relative to tunnel position separately for VR and DARK conditions (**Figure 5B, C**). In both cases licking occurred mostly at the end of the tunnel either before arrival at the reward zone or directly after reward delivery ("Start"). In DARK, however, there was more occasional licking throughout the tunnel. The number of rewards that mice collected were roughly similar across sessions (**Figure 5D**).

Analyzing these epochs of mouse behavior (running, turning, licking) during imaging allowed me to address the question of how spontaneous behavior shapes activity patterns in motor cortex. Another question which is gaining increasing interest in the field is how animals

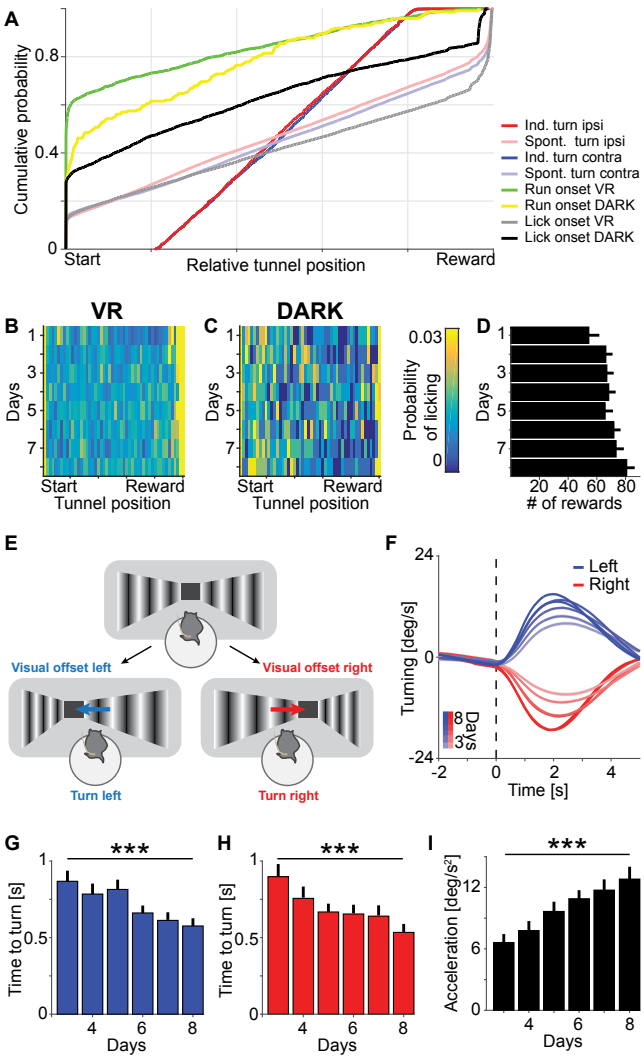

Figure 5. Quantification of mouse behavior

(**A**) Cumulative probability of occurrence of different behaviors as a function of relative position in the virtual tunnel. See detailed description in the main text. Pooled data from all mice which underwent imaging (n = 28). (**B, C**) Probability of licking as a function of relative position in the virtual tunnel either in VR condition (**B**) or in darkness (**C**). Mice preferentially licked around the time of reward delivery in VR conditions but occasionally also licked throughout the traversal when running in darkness. (**D**) Average number of rewards mice obtained over the course of eight days. Error bars are SEM over mice (n = 28). (**E**) Schematics of visual offset perturbations. The visual scenery was shifted by 30° either to the left or to the right in a randomized fashion requiring mice to perform a corrective turning movement. (**F**) Average turning speed in response to either a left (blue) or right (red) visual offset perturbation, for individual recording days. Shading is SEM over mice, n = 28. Time zero (dashed black line) marks stimulus onset. (**G, H**) Average time to significant turning onset in response to visual offsets for either left (**G**) or right turns (**H**), for each individual recording day. Error bars are SEM over mice, n = 28. ***, $p < 0.0005$. (**I**) Average acceleration during visual offset induced turns, for each individual recording day. Error bars are SEM over mice, n = 28. ***, $p < 0.0005$.

retain flexibility in goal-directed behavior under conditions of environmental uncertainty, that is: in the face of unexpected stimuli which require an adaptive response, the orchestration of which is presumed to be one of the major functions of motor cortex (Lopes et al., 2016).

To mimic such conditions in the head-fixed virtual reality task and to test for efficiency of turning behavior, I introduced, from day 3 on, visual offset perturbations which consisted of a 30° displacement of the visual scenery (**Figure 5E, see Movie_VisualOffsetPerturbations**). These visual offset perturbations occurred once per traversal with P = 0.8 overall probability, were restricted to 20% to 80% relative tunnel length (**Figure 5A**, induced turns), but otherwise random both in direction and exact location at which they would occur. Thus, mice could not form an expectation about the timing or direction of the stimulus. Accordingly, the optimal behavioral strategy which maximizes reward collection would be a straight running trajectory centered in the tunnel. Visual offset perturbations displaced the target and mice were required to perform a corrective turn in order to return to the ideal running trajectory through the virtual tunnel. Since I did not strictly reinforce the corrective turning movement itself (only whole tunnel traversals were rewarded), I first examined if mice changed their behavior in response to the visual offset perturbation. For each day, I aligned the turning behavior of the mice to the onset of the stimulus and found that on average for both offsets to the left or right, mice respond by vigorously turning already at the first day of testing (**Figure 5F**). Subsequently, I will use a descriptor that is independent of recording site: Contraversive turns are turns which occur in the direction contralateral to the recording site and, conversely, ipsiversive turns are those occurring in the direction ipsilateral to the recording site. Since recordings were exclusively performed in the right hemisphere, a contraversive turn corresponds to a left turn whereas an ipsiversive turn corresponds to a right turn. A corrective turning event lasted approximately 4 s. This behavior was learning-dependent since 1) the vigor of the behavior as measured by the turning velocity systematically increased (**Figure 5F**), 2) the reaction time measured as the time from stimulus to first significant behavioral change systematically decreased for both contra- and ipsiversive turns (**Figure 5G, H**, $p < 2 * 10^{-4}$, paired Student's t-test, n = 28 mice) and 3) acceleration increased over time (**Figure 5I,** $p < 10^{-6}$, paired Student's t-test, n = 28 mice). Thus, in response to unexpected visual stimuli, mice learn to efficiently execute corrective turning behavior.

There is substantial debate in the field of motor control as to what role motor cortex plays at what instance of motor behavior. Lesion studies in rodents have found impairments during skilled reaching control with much controversy prevailing as to the extent motor cortex is important during learning and / or execution of motor tasks (Guo et al., 2015; Kawai et al., 2015; Otchy et al., 2015). Additionally, it is often implicitly assumed that motor cortex does not play a role in seemingly simple movements such as running behavior. I challenged that view and manipulated the activity of frontal cortex corresponding to motor areas while animals were actively performing the navigation task.

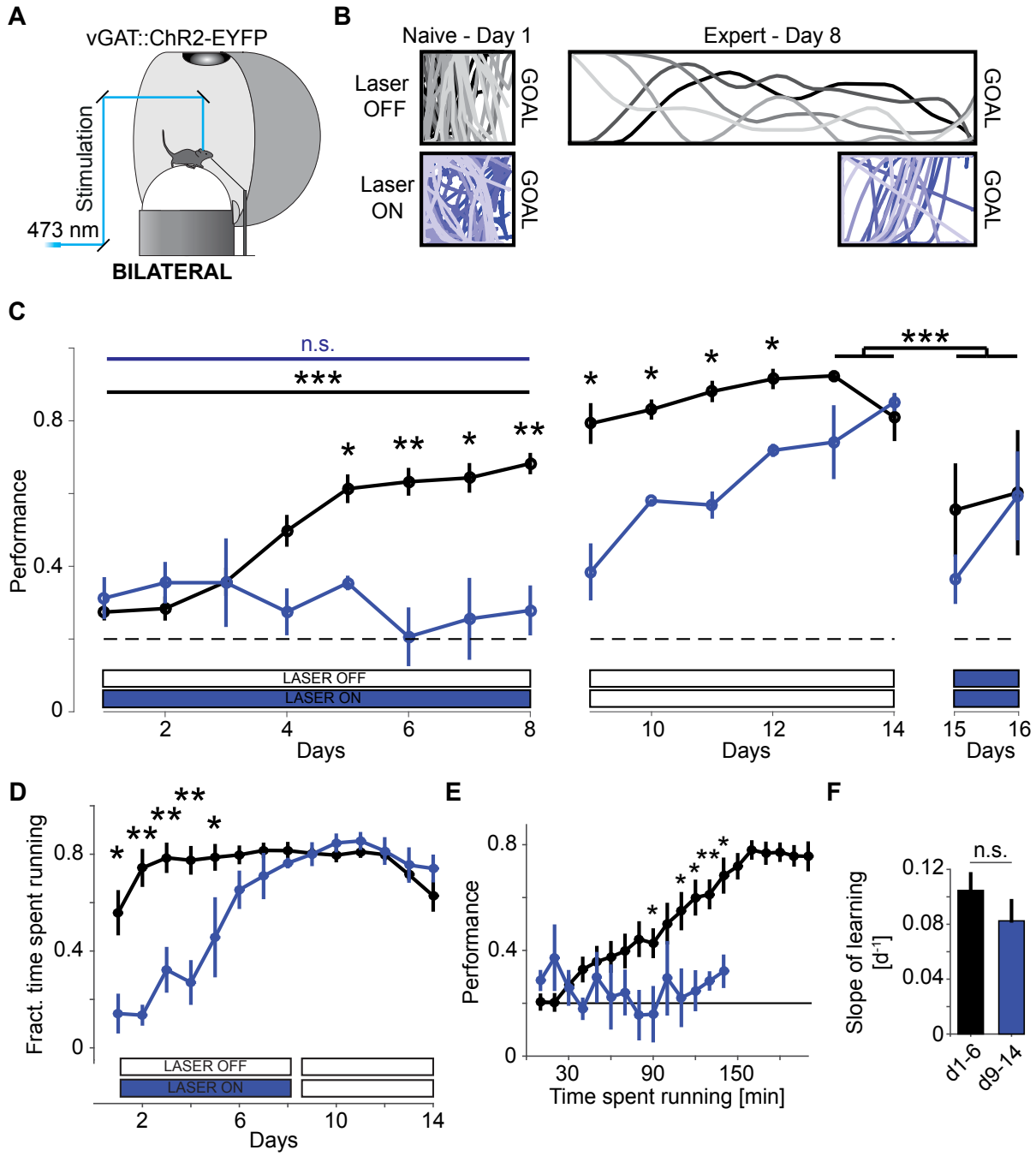

Figure 6. Motor cortex is required for visually guided navigation

(**A**) Schematic of experimental setup, as previously described but with 473 nm laser stimulation for optogenetic inhibition. (**B**) Schematic top-down view of virtual tunnel with individual example trajectories (in different hues) for either control mice on day 1 of training (top left, black hue), mice receiving photoinhibition at day 1 of training (bottom left, blue hue), control mice at day 8 of training (top right, black hue) and mice receiving photoinhibition at day 8 of training (bottom right, blue hue). Target zone is aligned at the right margin. Note I incrementally increased tunnel length when mice were collecting more than 1 reward per minute. Also note mice turning seemingly randomly on first training days and mice that received photoinhibition also do so late in learning.

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(**C**) Average performance over days as measured by the time spent running towards the target (see Experimental Procedures). Control group, black. Photoinhibition group, blue. Open and blue filled bars at the bottom indicate laser stimulation. Dashed black line indicates chance level. Error bars are SEM over mice (numbers indicated in the main text). *, **, ***, $p < 0.05$, 0.005, 0.0005. (**D**) Average fraction of time spent running, over days. Control group, black. Photoinhibition group, blue. Open and blue filled bars at the bottom indicate laser stimulation. Error bars are SEM over mice (numbers indicated in the main text). *, **, $p < 0.05$, 0.005. (**E**) Average performance normalized by time spent running in the first 8 days of recording. Control group, black. Photoinhibition group, blue. Solid black line indicates chance level. Error bars are SEM over mice . *, **, $p < 0.05$, 0.005. (**F**) Average slope of learning for control mice (black, n = 14) and mice that received photoinhibition (blue, n = 3) measured over the indicated days (see Experimental Procedures). Error bars are SEM over mice.

5.2 Virtual navigation requires motor cortex

5.2.1 Motor cortex is necessary for visually guided virtual navigation

To perform targeted inhibition of motor cortex I implanted cranial windows bilaterally over both left and right motor cortices in transgenic mice that express channelrhodopsin-2 in all γ-aminobutyric acid-positive (GABAergic) inhibitory interneurons (vGAT::ChR2(H134R)-EYFP; JAX #014548). In a subset of mice, I then directed a laser beam (473nm) at motor cortex in the first eight training sessions while mice navigated through the virtual tunnel (**Figure 6A**, see Experimental Procedures). Mice were trained either with motor cortex inhibited (3 mice, blue) or under non-inhibited control conditions (14 mice, black). Individual trajectories indicated mice were initially not able to efficiently reach the goal either because they were not able to control the styrofoam ball or have not formed the association between target and reward yet (**Figure 6B**). This was the case for both groups of mice. I defined a task performance measure which quantified the time mice were running either in the direction of the target or running facing away from the target. I chose an arbitrary angle of 72° heading which resulted in 20% chance performance (see Experimental Procedures). Without motor cortex inhibition, mice significantly increased their performance over the course of eight days of training such that trajectories became goal-directed (**Figure 6B**, black). When motor cortex was inhibited during training, mice failed to improve in task performance: They continued turning most of the time. (**Figure 6B**, blue). I then quantified the performance of the mice (**Figure 6C**, see Experimental Procedures). After 8 days of training, control mice performed significantly better than at the beginning of training ($p < 10^{-6}$, paired Student's t-test, n = 14 mice). In contrast, mice which received photoinhibition in motor cortex failed to learn the task (**Figure 6C,** $p > 0.9$, paired Student's t-test, n = 3 mice). Differences in

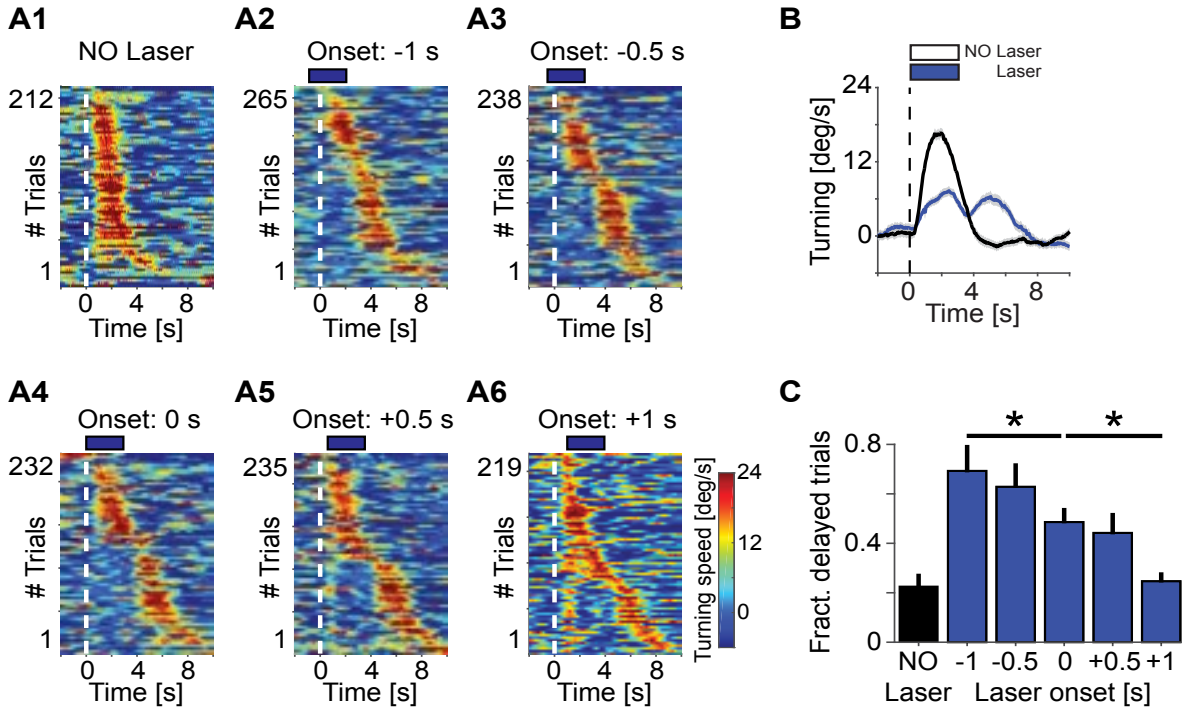


Figure 7. Motor cortex is required for visual perturbation induced corrections of motor control

(**A**) Corrective induced turning in response to individual perturbation trials either without (**A1**, control) or with bilateral photoinhibition of motor cortex (**A2 – A6**, photostimulus onset times as indicated in the respective panel). Photoinhibition impaired the likelihood of mice initiating a corrective turn. Time zero (white dashed line) marks onset of perturbation stimulus. Blue bar above panel marks duration of photostimulus. (**B**) Average turning speed during either no photoinhibition (same data as in **A1**, black) or photoinhibition concurrent with onset of perturbation stimulus (same data as in **A4**, blue). Shading indicates SEM over trials. Time zero (dashed black line) marks onset of perturbation stimulus. Blue bar marks duration of photostimulus. (**C**) For every condition shown in (**A**) fraction of trials in which induced turning in response to the perturbation stimulus was delayed (see Experimental Procedures). Error bars are SEM over mice (n = 5). *, $p < 0.05$.

running levels could account for the differences in performance I observed. Indeed, while both groups of mice were able to move, the overall fraction of time spent running was initially lower for mice which received photoinhibition (**Figure 6D**, days 1 – 5, $p < 0.05$, 0.005, 0.005, 0.05, unpaired Student's t-test, in n = 14 and 3 mice, respectively). In order to account for this potential confound, I quantified performance binned by time spent running and found that also with this measure, mice which received photoinhibition were impaired compared to control mice, ruling out an inability to move as the reason of impaired task performance (**Figure 6E**, bins 110 - 140 minutes, $p < 0.05$, 0.05, 0.005, 0.05, unpaired Student's t-test, n = 14 and 3 mice, respectively).

I then continued training both groups of mice (3 mice previously trained with motor

cortex inhibition and 3 mice without motor cortex inhibition) for another six training sessions without inhibition of motor cortex (**Figure 6C**). Performance in mice that had received no inhibition of motor cortex in the initial eight training sessions remained largely unchanged but mice that received inhibition of motor cortex in the initial eight training sessions increased their performance to a performance level matching that of normally trained mice (**Figure 6C**, day 13, control vs photoinhibition group, $p > 0.05$, paired Student's t-test, n = 3 mice). Moreover, the slope of learning was not different whether mice received photoinhibition previously or not (**Figure 6F**, $p > 0.4$, unpaired Student's t-test, n = 14 and 3 control and photoinhibition mice, respectively). Acute inhibition of motor cortex in training sessions 15 and 16 then resulted in significant decreases in performance in both groups of mice (**Figure 6C**, $p < 0.0005$, paired Student's t-test, n = 5 mice). These results are consistent with the interpretation that neural activity in motor cortex is necessary for motor control during virtual navigation.

5.2.2 Motor cortex is necessary for visual perturbation induced corrections of motor control

To test the involvement of motor cortical neural activity in motor corrections in response to unpredictable visual offset perturbations, I timed, in trained mice (at least 35 training sessions), the onset of the photoinhibition to the visual offset perturbations (**Figure 7**). Six different trial types consisting of either no photoinhibition (**Figure 7A1**, control) or photoinhibition prior to, concurrent with or following the visual offset perturbation (**Figure 7A2 - 3, A4** or **A5 – 6**, respectively) were chosen. Photoinhibition lasted 3 s and trials were randomly interleaved to prevent mice from predicting the timing of photoinhibition. Average traces indicated that inhibition of motor cortex delayed corrective turning responses relative to control conditions (**Figure 7B**). The fraction of trials without corrective turns systematically depended on the timing of the onset of the motor cortex inhibition (**Figure 7C,** -1 s vs 0 s laser onset, 0 s vs +1 s laser onset, $p < 0.05, 0.05$, paired Student's t-test, n = 5 mice). When motor cortex inhibition preceded the visual offset perturbation (**Figure 7A2**, **C**, -1 s onset), the fraction of trials without corrective response was significantly larger than with concurrent activation (**Figure 7A4, C,** $p < 0.05$, paired Student's t-test, n = 5 mice); conversely, when inhibition onset followed the visual perturbation (**Figure 7A6**, 1 s delay, $p < 0.005$, n = 5 mice), the fraction of trials without corrective response was significantly smaller than with concurrent activation (**Figure 7C**). These results are consistent with an interpretation in which ongoing neuronal activity in motor cortex determines the implementation of subsequent motor programs.

Interestingly, trials with photoinhibition were either on time or fully delayed with few intermittent turning suggesting that motor programs would either be executed as intended or completely erased (**Figure 7A**).

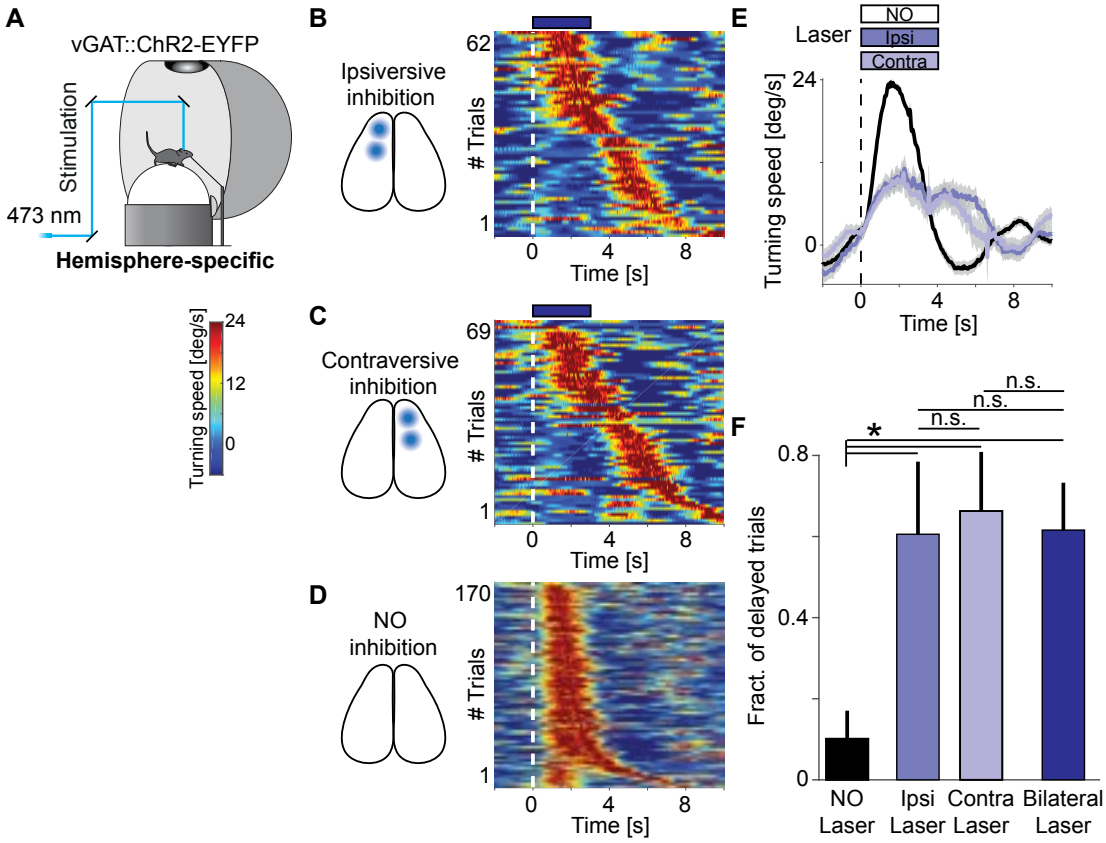


Figure 8. Unilateral optogenetic inhibition impairs corrective turning behavior

(**A**) Schematic of the experimental setup, identical to Figure 7, but laser stimulation was directed at only one hemisphere at a time. (**B**) Corrective induced turning trials with photoinhibition during ipsiversive turns. Time zero (dashed white line) marks onset of visual offset stimulus. Blue bar indicates duration of the photostimulus. (**C**) Same as (**B**) but for photoinhibition during contraversive turns. (**D**) Same as (**B**) but without photoinhibition. (**E**) Average turning speed during either no photoinhibition (same data as in (**D**), black) or photoinhibition concurrent with onset of perturbation stimulus during either ipsi- or contraversive turns (dark and pale blue, respectively, average data for (**B**) and (**C**)). Shading indicates SEM over trials. Time zero (dashed black line) marks onset of perturbation stimulus. Blue bar marks duration of photostimulus. (**F**) For conditions shown in (**B - D**) fraction of trials in which induced turning in response to the perturbation stimulus was delayed (see Experimental Procedures). Location of photostimulus relative to turn direction as indicated below panel. Data for bilateral photostimulation is identical to **Figure 7A4**. Error bars are SEM over mice (n = 5). *, $p < 0.05$.

5.2.3 Unilateral optogenetic inhibition is as effective as bilateral inhibition

A recent study has shown that the unaffected cortical hemisphere can restore neuronal activity patterns in the perturbed hemisphere to levels that behavioral phenotypes were also ameliorated

(Li et al., 2015b) and lateralization of motor control, at least in humans, has been a long-standing hypothesis (Mutha et al., 2012).

In order to test for the possibility of lateralized contributions in the corrective turning response to the visual offset perturbation, I performed an identical experiment as described in the previous section (**Figure 7**) but now inhibited motor cortical areas hemisphere-specificly (**Figure 8A**). Concurrent with visual perturbation onset, I photoinhibited either left motor cortex during left turns and right motor cortex during right turns and pooled the data (**Figure 8B**, ipsiversive inhibition) or photoinhibited left motor cortex during right turns and right motor cortex during left turns and pooled the data (**Figure 8C**, contraversive inhibition). In the control condition, photoinhibition was omitted (**Figure 8D**). When I aligned the turning behavior to visual perturbation onset for each condition, there was no difference between ipsiversive or contraversive inhibition (**Figure 8B – E)**. However, the fraction of trials in which corrective turning was delayed was significantly higher for either ipsi- or contraversive photoinhibition condition compared to the control condition (**Figure 8F**, $p < 0.05$, paired Student's t-test, $n = 5$ mice). Moreover, the fraction of delayed trials during ipsi- or contraversive photoinhibition was not different (**Figure 8F**, $p > 0.3$, paired Student's t-test, $n = 5$ mice) and either unilateral photoinhibition condition was not different from bilateral inhibition (**Figure 8F**, $p > 0.4$, paired Student's t-test, $n = 5$ mice, bilateral data is identical to Figure 7, 0 s onset condition). Thus, both unilateral and bilateral motor cortex inhibition impaired mice to a similar degree suggesting that essential computations for motor control are taking place in either hemisphere.

5.2.4 Channelrhodopsin mediates impairment during optogenetic inhibition

Impairments in the ability to initiate corrective turning movements could be due to startle or laser light being visible to the mice. To account for the latter, a study using optogenetic inhibition employed a very bright (blinding) light in order to equalize illumination levels during control conditions and laser stimulation conditions (Guo et al., 2014). The task I trained the mice in requires visually guided behavior which precludes blinding illumination levels. To minimize changes in illumination due to the laser being switched on for photostimulation which might allow mice to predict photoinhibition epochs, the laser was cycled between two off-target positions at the back of the mice's skull, over the dental cement, during the periods of laser off (see Experimental Procedures). This arrangement had the added advantage that noise levels derived from the scanning mirrors also did not change audibly anymore when the laser was switched on-target.

Another potential source of concern could be light penetration through the brain, back-illuminating the retina which could interfere with visually guided behavior. Stray laser light could be measured exiting through the eye by a conventional charge-coupled device (CCD)

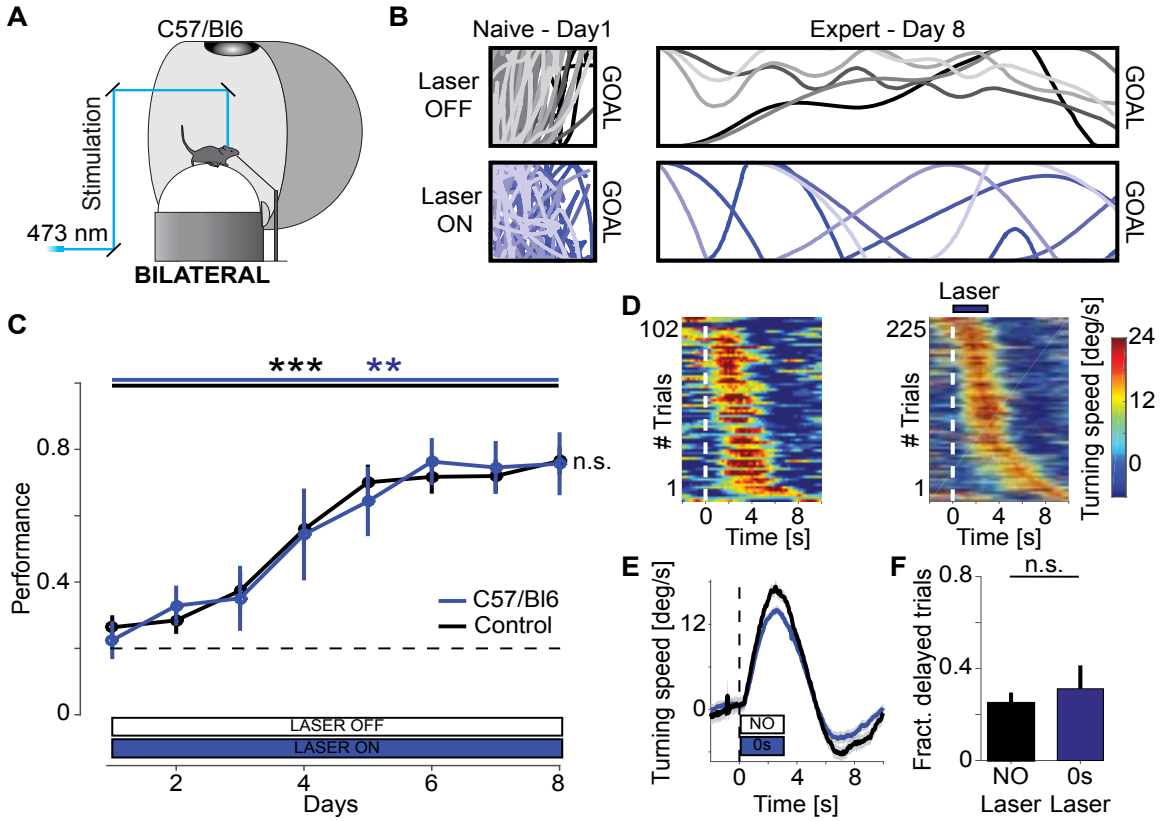


Figure 9. Channelrhodopsin mediates impairment during optogenetic inhibition

(**A**) Schematic of the experimental setup, identical to Figure 6, except mice were of C57/Bl6 genotype. (**B**) Schematic top-down view of virtual tunnel with individual example trajectories (in different hues) for either control mice on day 1 of training (top left, black hue), C57/Bl6 mice receiving photoinhibition at day 1 of training (bottom left, blue hue), control mice at day 8 of training (top right, black hue) and C57/Bl6 mice receiving photoinhibition at day 8 of training (bottom right, blue hue). Target zone is aligned at the right margin. Note I incrementally increased tunnel length when mice were collecting more than 1 reward per minute. Also note mice turning seemingly randomly on first training days. Data of control mice is identical to Figure 6. (**C**) Average performance over days as measured by the time spent running towards the target (see Experimental Procedures). Control group, black, same data as Figure 6 days 1 - 8 (n = 14). C57/Bl6 photoinhibition group, blue. Open and blue filled bars at the bottom indicate laser stimulation. Dashed black line indicates chance level. Error bars are SEM over mice. **, ***, $p < 0.005, 0.0005$. (**D**) Corrective induced turning trials without (left panel) and with photoinhibition concurrent with visual offset stimulus (right panel). Time zero marks onset of visual offset stimulus. Blue bar indicates duration of the photostimulus. (**E**) Average turning speed during either no photoinhibition (same data as in (**D,** left), black) or photoinhibition concurrent with onset of perturbation stimulus (same data as in (**D**, right), blue). Shading indicates SEM over trials. Time zero marks onset of perturbation stimulus. Blue bar marks duration of photostimulus. (**F**) For conditions shown in (**D**) fraction of trials in which induced turning in response to the perturbation stimulus was delayed (see Experimental Procedures). Error bars are SEM over mice (n = 5).

camera (data not shown). To address this concern, I trained mice lacking the channelrhodopsin allele under identical conditions (**Figure 9A**). Performance of C57/Bl6 mice which received photostimulation (experimental group) was indistinguishable from control mice which did not receive photoinhibition (**Figure 9B, C**). Mice learned visually guided motor behavior resulting in goal-directed traversals through the virtual tunnel (**Figure 9B**) and quantification of performance showed significant learning of both groups to similar expertise (**Figure 9C**, day 1 vs day 8, control and C57/Bl6 experimental, $p < 10^{-6}$ and 0.005, paired Student's t-test, day 8 control vs experimental, $p > 0.9$, unpaired Student's t-test, n = 14 and 5 mice, respectively). Thus, the impairment in learning observed in previous experiments (**Figure 6**) is due to the presence of channelrhodopsin.

I then subjected the trained C57/Bl6 mice to timed photoinhibition concurrent with visual offset perturbation stimuli. I aligned the turning behavior to perturbation onset and found no obvious difference between non-photoinhibited and photoinhibited trials (**Figure 9D, E**). The fraction of delayed trials between the two conditions was not significantly different (**Figure 9F,** $p > 0.1$, paired Student's t-test, n = 4 mice) showing that the presence of channelrhodopsin in GABAergic interneurons is required for the impairment in initiating corrective turning behavior seen during earlier optogenetic manipulations.

Taken together, the data derived from optogenetic inhibition experiments are consistent with the interpretation that activity in motor cortex is necessary for the execution of corrective movements driven by unpredictable visual feedback.

5.3 Two-photon calcium imaging in identified cell types

5.3.1 Imaging in motor cortex

I established that motor cortex is required for learning and execution of the virtual navigation task. I subsequently attempted to understand what activity patterns might underlie the motor control programs employed by mice during virtual navigation and how they are shaped by experience. In many mouse brain areas, functional cell classes were reported to be intermingled, e.g. in rat visual cortex, cells selective for orthogonal directions could be found in close vicinity (Ohki et al., 2005), in mouse barrel cortex, cells located in the same field of view were recruited in a mouse behavior-dependent fashion (Chen et al., 2013) and in mouse motor cortex, the correlation between function and location was only weak (Dombeck et al., 2009). Function of a cell in a microvolume of brain tissue is therefore relatively poorly predicted by location but mounting evidence suggests that rather connectivity is a major determinant (Cossell et al., 2015). All of these studies employed two-photon calcium imaging as the current method of choice to infer activity of tens (up to hundreds) of neurons near-simultaneously in a small volume of the brain.

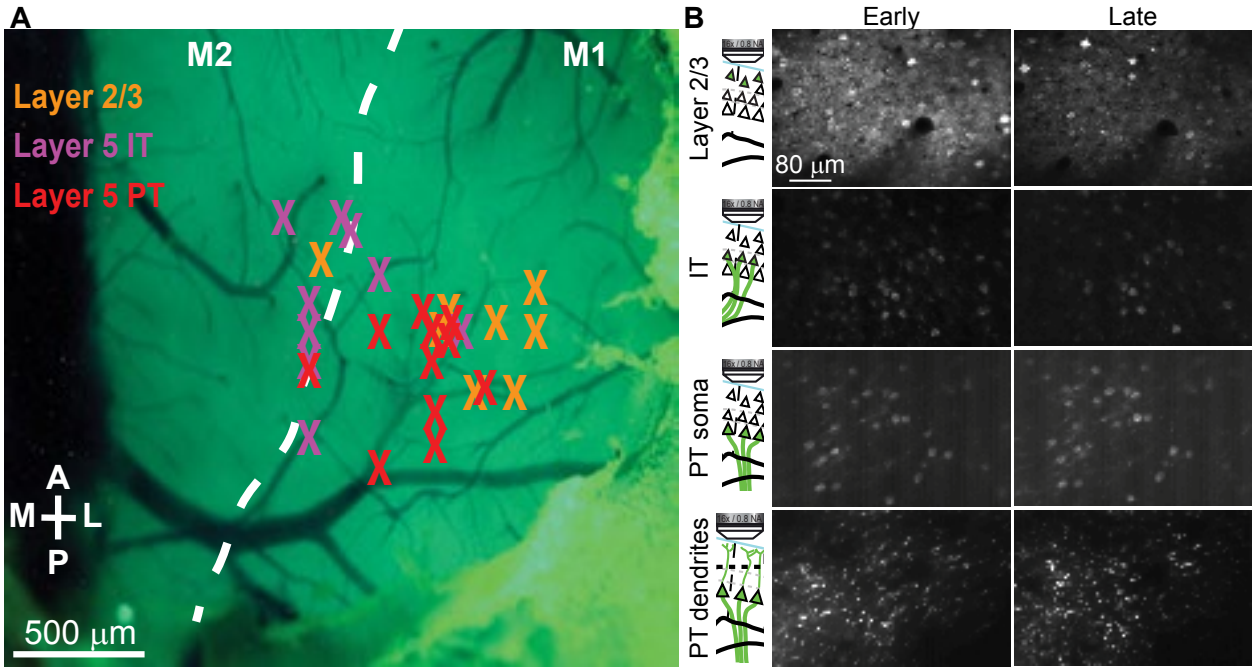


Figure 10. Chronic two-photon calcium imaging of genetically defined cell types

(**A**) Top-down view on the cortical surface of a mouse implanted with a cranial window with center of injection sites superimposed for individual mice. Layer 2/3 recording sites marked in orange, layer 5 IT in purple and layer 5 PT in red. White cross indicates location of bregma. Scale bar as indicated. M1, primary motorv cortex. M2, secondary motor cortex as delineated in (Paxinos and Franklin, 2001). (**B**) Example field of view for either layer 2/3, layer 5 IT, layer 5 PT soma or layer 5 PT dendrites recordings (from top to bottom row) either early (left column, days 1 – 4) or late (right column, days 5 – 8) in learning.

Using two-photon calcium imaging, I recorded neural activity in motor cortex chronically throughout learning while mice were actively behaving and performing the task in three major cell types: layer 2/3 neurons, layer 5 IT neurons (Tlx3-positive) and layer 5 PT (Sim1-positive) neurons (**Figure 10**). I used adeno-associated viral (AAV) vectors to stably transfect different cell types with the genetically-encoded calcium indicator GCaMP6f (Chen et al., 2013). For recordings of layer 2/3 neurons I used an AAV2/1-EF1α-GCaMP6f virus to express GCaMP6f in all neurons. For the layer 5 recordings I used an AAV2/1-EF1α-DiO-GCaMP6f in either Tlx3-Cre mice (layer 5 IT) or Sim1(KJ18)-Cre mice (layer 5 PT) (Gerfen et al., 2013) to express GCaMP6f specificly in either layer 5 IT or layer 5 PT neurons. In a subset of mice, I recorded from layer 5 PT neuron apical dendrites. Data obtained from recording either layer 5 PT dendrites or soma were qualitatively comparable (**Figure S1**). I therefore pooled both data sets in all subsequent analyses.

Injections were centered at A-P +0.5, M-L 1.5 in primary motor cortex (**Figure 10**), a region which when mapped by ICMS, has previously been reported to be part of the caudal forelimb area (Tennant et al., 2011), was located slightly more medial to sites where movement-related activity has been recorded by similar methods (Dombeck et al., 2009) but was distant to what has been referred to as anterior lateral motor (ALM) cortex shown to be involved in licking motor behavior (Guo et al., 2014; Komiyama et al., 2010; Li et al., 2015a).

5.3.2 Activation during spontaneous turns linearly scales with motor behavior

To understand activity patterns in mouse motor cortex involved in navigation and corrective turning behavior, I analyzed events in which mice were spontaneously turning as they traversed the virtual tunnel. For most of the subsequent results, activity was analyzed separately for the three different cell classes.

First, I quantified the activity of layer 2/3, layer 5 IT and layer 5 PT neurons as a function of the amplitude of the turn (**Figure 11A**). Based on the measured behavior of the mice, I selected times when mice spontaneously initiated an ipsiversive or a contraversive turn (see Experimental Procedures; **Figure 11B**). I classified neurons as either responsive during ipsiversive turns or responsive during contraversive turns (see Experimental Procedures). The activity of individual cells was dependent on the vigor of the turning (**Figure S2**). I then quantified the mean response of ipsiversive neurons to ipsiversive turns and the response of contraversive neurons to contraversive turns as a function of the speed of the turn. I found that the activity of neurons in layer 2/3 and layer 5 IT and layer 5 PT all scaled linearly with the speed of the turn (**Figure 11C, D**, first vs last bin, $p < 10^{-55}$, 10^{-12}, 10^{-9}, paired Student's t-test and $p < 10^{-113}$, 10^{-29}, 10^{-25}, linear trend analysis, for layer 2/3 (**D1**, n = 1154 neurons), layer 5 IT (**D2**, n = 308 neurons) and layer

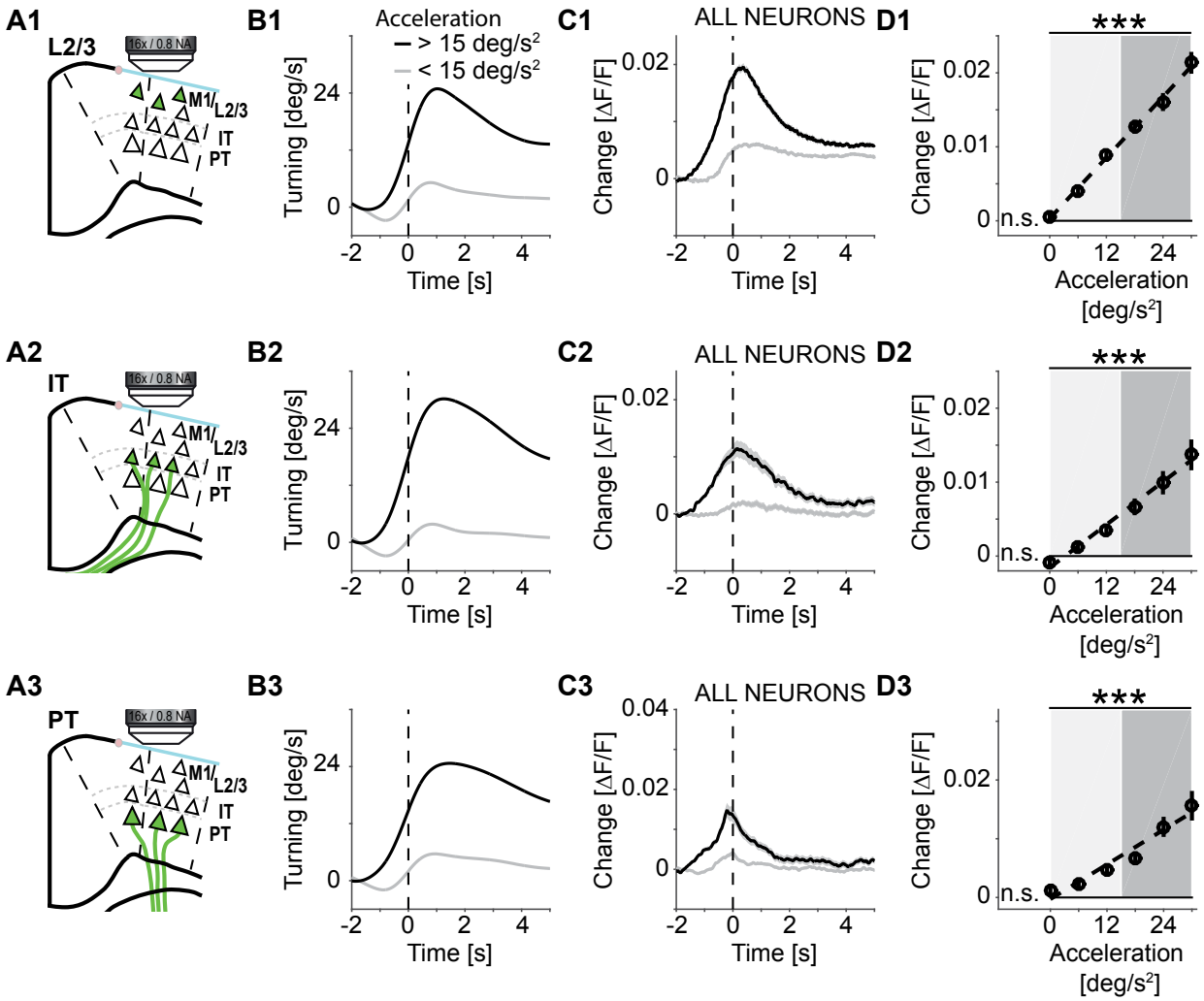


Figure 11. Activation during spontaneous turns scales linearly with motor behavior

(**A**) Schematic recording configuration for layer 2/3 (**A1**), layer 5 IT (**A2**) and layer 5 PT (**A3**). (**B**) Average spontaneous turning speed in low (acceleration < 15 deg/s^2, grey) and high (acceleration > 15 deg/s^2, black) magnitude of turning bins. Shading indicates SEM over trials. Time zero (dashed black line) marks onset of turn. (**C**) Average activity during spontaneous turns binned as in (**B**). Shading indicates SEM over neurons (n = 1154, 308 and 560 in layer 2/3 (**C1**), layer 5 IT (**C2**) and layer 5 PT (**C3**), respectively). Time zero (dashed black line) marks onset of turn. Note increase in activity as mice turn faster. (**D**) Average activity during spontaneous turning events of different acceleration. Error bars indicate SEM over neurons (n = 1154, 308 and 560 in layer 2/3 (**D1**), layer 5 IT (**D2**) and layer 5 PT (**D3**), respectively). Solid black line indicates 0 change [ΔF/F]. Dashed black line indicates a line fit to the data. Grey shading indicates bins used to generate average turning speed and activity trace in (**B**) and (**C**). ***, $p < 0.0005$. Activity in zero bin was not significantly different from 0.

5 PT (**D3**, n = 560 neurons), respectively). There was no significant activation when mice were not spontaneously turning (**Figure 11D**, zero bin, $p > 0.05$, Student's t-test for zero mean, for all

cell types). Thus, during spontaneous turning events, activation throughout local motor cortex was linearly dependent on the turning speed of the mice. This is consistent with the general assumption that the amount of motor-related activity in the brain determines the vigor of motor behavior.

The vigor of corrective turning behavior systematically increased in the course of learning (see **Figure 5 F – I**). It could be that the linear scaling of activity by spontaneous motor behavior is also experience-dependent. To address this question, I analyzed turning speed-dependent neuronal activation in the three cell types (**Figure 12A)** separately for early (**Figure 12B**, training days 1 – 4) and late (**Figure 12C**, training days 5 – 8) sessions. Regardless of the learning stage, I found that activity in both layer 2/3 and layer 5 scaled linearly with the spontaneous motor behavior (F**igure 12B, C,** first vs last bin early sessions, $p < 10^{-54}$, 10^{-10}, 10^{-12}, paired Student's t-test and $p < 10^{-111}$, 10^{-19}, 10^{-26}, linear trend analysis, first vs last bin late sessions, $p < 10^{-37}$, 10^{-10}, 10^{-9}, paired Student's t-test and $p < 10^{-70}$, 10^{-24}, 10^{-21}, linear trend analysis, for layer 2/3 (**B1, C1**, n = 1154 neurons), IT (**B2, C2**, n = 308 neurons) and PT (**B3, C3**, n = 560 neurons), respectively)

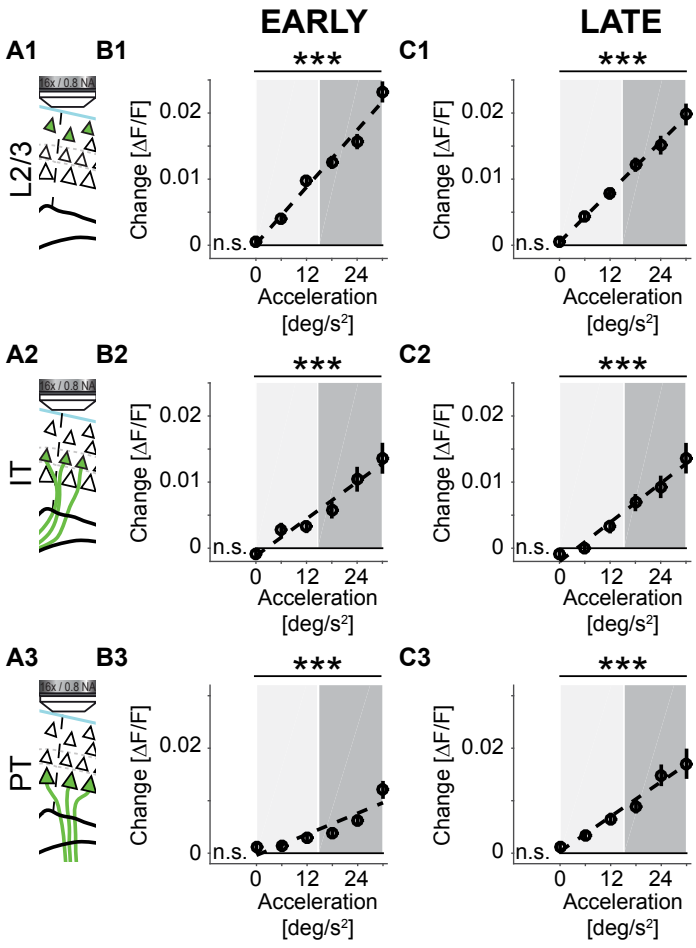


Figure 12. Activation during spontaneous turns linearly scales with motor behavior early and late during learning

(**A**) Schematic recording configuration for layer 2/3 (**A1**), layer 5 IT (**A2**) and layer 5 PT (**A3**). (**B**) Average activity during spontaneous turning events of different acceleration during early learning days 1-4. Error bars indicate SEM over neurons (n = 1154, 308 and 560 in layer 2/3 (**B1**), layer 5 IT (**B2**) and layer 5 PT (**B3**), respectively). Solid black line indicates 0 change [ΔF/F]. Dashed black line indicates a line fit to the data. Grey shading indicates bins contributing to generate average turning speed and activity trace in (**Figure 11B**) and (**Figure 11C**). ***, $p < 0.0005$. Activity in zero bin was not significantly different from 0. (**C**) Same as in (**B**) but for late learning days 5-8.

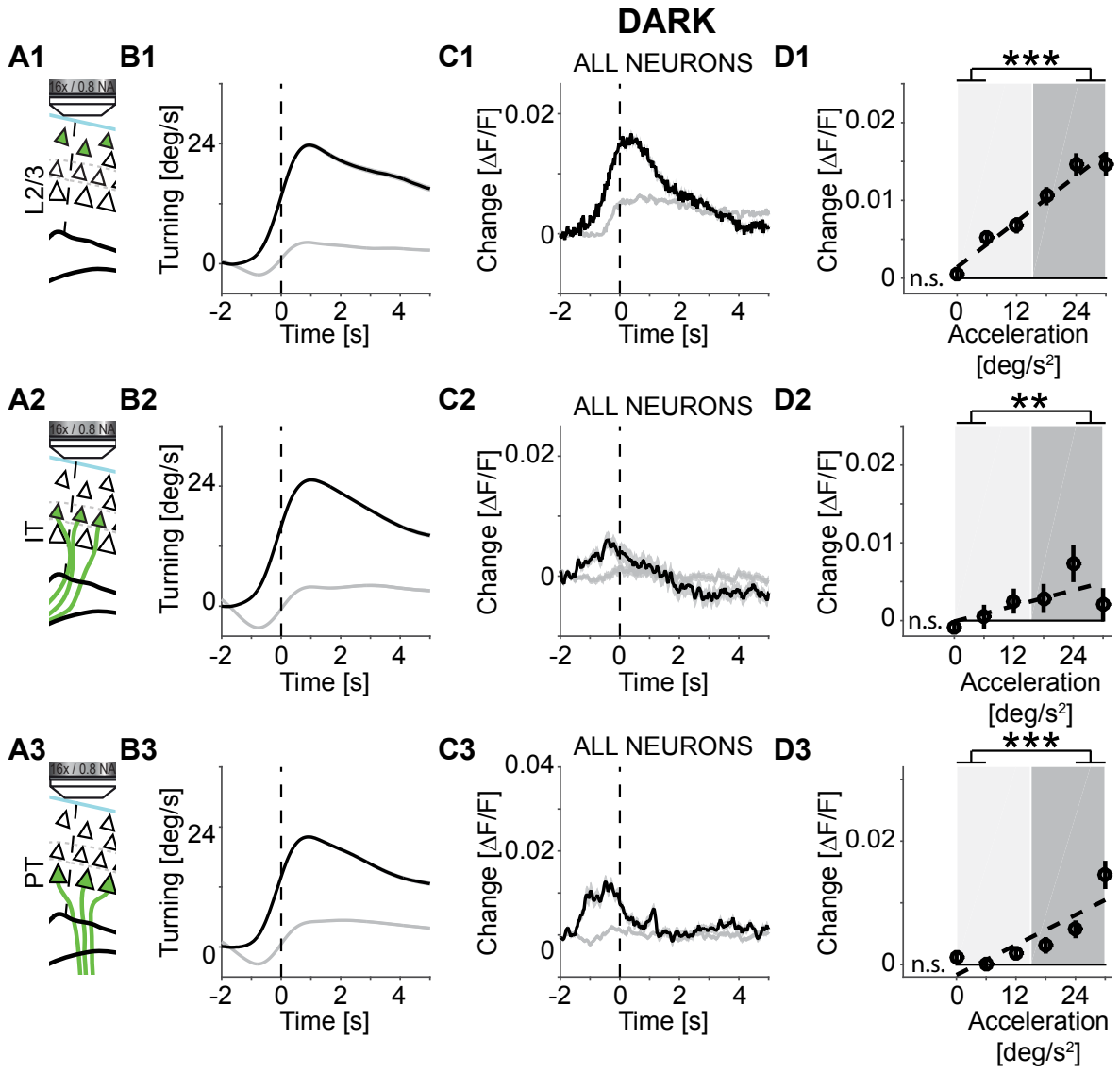


Figure 13. Activation during spontaneous turns in darkness linearly scales with motor behavior

(**A**) Schematic recording configuration for layer 2/3 (**A1**), layer 5 IT (**A2**) and layer 5 PT (**A3**). (**B**) Average spontaneous turning speed in darkness in low (acceleration < 15 deg/s^2, grey) and high (acceleration > 15 deg/s^2, black) magnitude of turning bins. Shading indicates SEM over trials. Time zero (dashed black line) marks onset of turn. (**C**) Average activity during spontaneous turns binned as in (**B**). Shading indicates SEM over neurons (n = 1154, 308 and 560 in layer 2/3 (**C1**), layer 5 IT (**C2**) and layer 5 PT (**C3**), respectively). Time zero (dashed black line) marks onset of turn. Note increase in activity as mice turn faster. (**D**) Average activity during spontaneous turning events of different acceleration. Error bars indicate SEM over neurons (n = 1154, 308 and 560 in layer 2/3 (**D1**), layer 5 IT (**D2**) and layer 5 PT (**D3**), respectively). Solid black line indicates 0 change [ΔF/F]. Dashed black line indicates a line fit to the data. Grey shading indicates bins used to generate average turning speed and activity trace in (**B**) and (**C**). **, ***, $p < 0.005, 0.0005$. Activity in zero bin was not significantly different from 0.

and activation was only measureable when mice were actively turning (**Figure 12B, C**, zero bin, $p > 0.05$, Student's t-test for zero mean, for all cell types in both conditions). Thus, the linear relationship of activity and behavior during spontaneous turns appears to be an innate feature of neuronal activation in motor cortex.

Lastly, I examined whether the presence of visual cues has an effect on the activity during spontaneous turning events. For this, I analyzed activity recorded during darkness (**Figure 13**). Data was noisier due to shorter recording time and activation was apparent at an earlier time relative to behavior onset compared to VR conditions (**Figure 13C**). I quantified the relationship between activity and motor behavior and found again that a linear model described the data well (**Figure 13D,** first two vs last two bins, $p < 10^{-32}$, 0.005, 10^{-12}, paired Student's t-test and $p < 10^{-40}$, 0.05, 10^{-19}, linear trend analysis, for layer 2/3 (**D1**, n = 1154 neurons), IT (**D2**, n = 308 neurons) and PT (**D3**, n = 560 neurons), respectively). Note this analysis was done using the same response windows as in previous figures which is underestimating the effect because of the temporal shift in activation.

In summary, I find that activation during spontaneous turning events is linearly dependent on the turning motor behavior (**Figure 11**). This effect was present irrespective of learning stage (early vs late, **Figure 12**) and whether mice were executing spontaneous turns with visual guidance or in darkness (**Figure 13**).

5.3.3 Activation during running onsets

Studies using electrophysiological methods in rabbits and cats found that activity in motor cortex layer 5 corticofugal neurons positively correlated with locomotion rhythm (Beloozerova et al., 2003b), postural control movements (Beloozerova et al., 2003a) and gait parameters (Drew et al., 2002). Moreover, a study using two-photon calcium imaging in mice found correlation between running behavior and recorded cellular activity in layer 2/3 (Dombeck et al., 2009). Consistent with the literature, during spontaneous turning events I have observed linear scaling of activity with motor behavior across a range of conditions. To investigate how motor cortex is active and how this activity is shaped by experience and directly compares to another type of movement, I analyzed activity during running onsets.

In order to extract epochs of running onsets, the forward velocity behavior was thresholded. Mice most likely initiated spontaneous locomotion after reward collection (**Figure 5A**). To avoid possible interference of the signal by slow offset dynamics of the calcium indicator generated by preceding events, I only considered running onsets when mice had been stationary and not rewarded for at least 5s (see Experimental Procedures). I aligned the activity to epochs during which the onset of running resulted in high speed and those in which mice ran at low speed (**Figure**

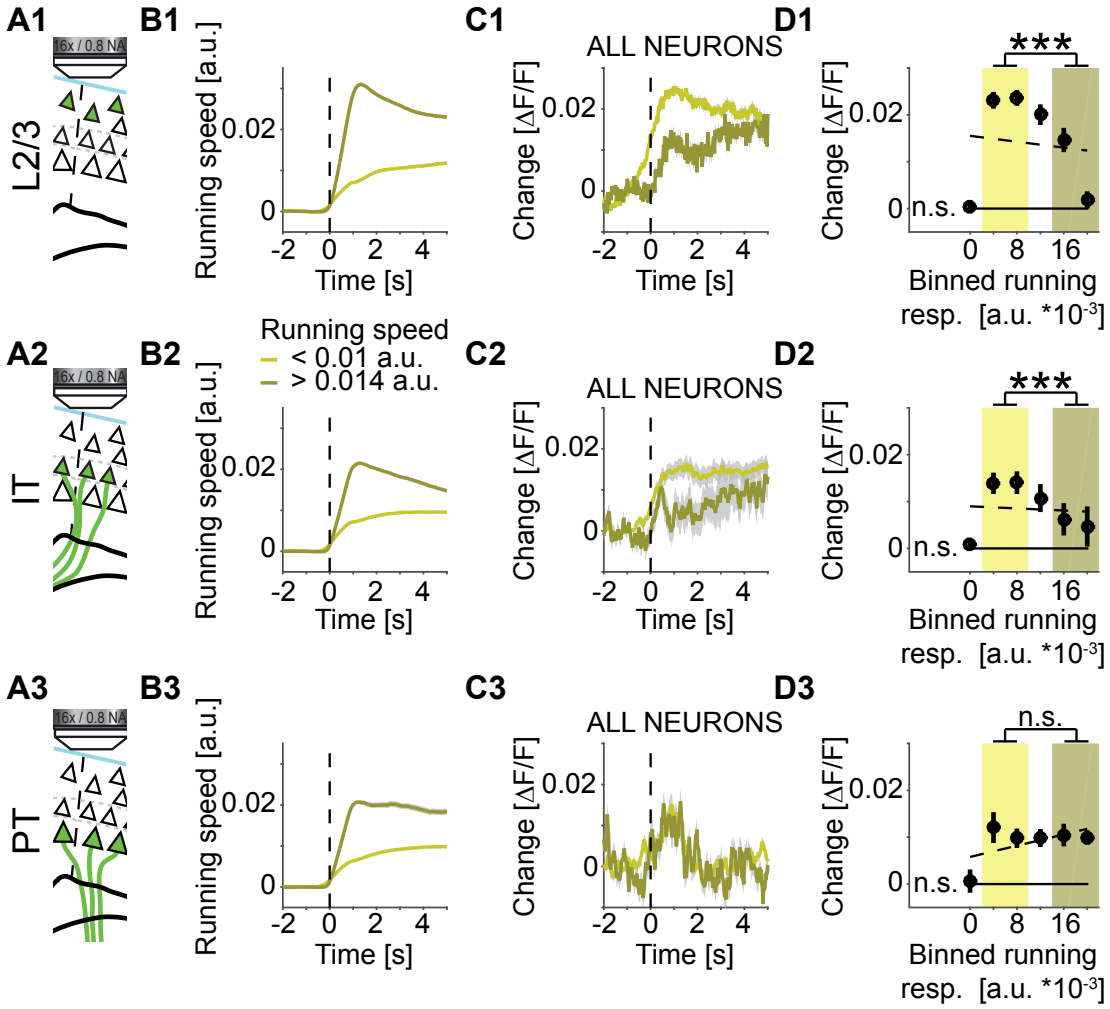


Figure 14. Activation during visually-guided running is higher at lower speeds

(**A**) Schematic recording configuration for layer 2/3 (**A1**), layer 5 IT (**A2**) and layer 5 PT (**A3**). (**B**) Average running speed during self-initiated locomotion in low (speed < 0.01 a.u., pale yellow) and high (speed > 0.014 a.u., dark yellow) magnitude of running bins. Shading indicates SEM over trials. Time zero (dashed black line) marks running onset. (**C**) Average activity during running onsets binned as in (**B**). Shading indicates SEM over neurons (n = 1154, 308 and 560 in layer 2/3 (**C1**), layer 5 IT (**C2**) and layer 5 PT (**C3**), respectively). Time zero (dashed black line) marks running onset. (**D**) Average activity during running onset events of different speed. Error bars indicate SEM over neurons (n = 1154, 308 and 560 in layer 2/3 (**D1**), layer 5 IT (**D2**) and layer 5 PT (**D3**), respectively). Solid black line indicates 0 change [ΔF/F]. Dashed black line indicates a line fit to the data. Shading indicates bins used to generate average running speed and activity trace in (**B**) and (**C**). ***, 0.0005. Activity in zero bin was not significantly different from 0.

14B, C). In layer 2/3, activity during low speed running already rose before the onset of behavior (**Figure 14C1**). This ramp up of activity had previously been observed and termed preparatory activity (Churchland et al., 2006; Churchland and Shenoy, 2007). Preparatory activity was less

apparent in layer 5 (but see section on preparatory activity for a more elaborate analysis). I then assessed activity as a function of speed of the subsequent running epoch (**Figure 14D**). Activity was not present during epochs of no running (**Figure 14D**, $p > 0.1$ for all cell types, Student's t-test for zero mean, n = 1154, 308 and 560 neurons **(D1 – D3)**, respectively). In contrast to spontaneous turn events in which activity scaled linearly with mouse behavior, I found a linear relationship only for running onset-related activity in layer 5 PT neurons (**Figure 14D1 – D3**, $p > 0.3$, 0.7 and $p < 0.05$, linear trend analysis, n = 1154, 308 and 560 neurons, respectively). Moreover, I found that activity was highest at the lowest speed bin different from zero. Activity was either less for higher running speeds in layer 2/3 and IT (**Figure 14D1, D2**, 2nd and 3rd bin vs 2nd last and last bin, $p < 10^{-12}$, 10^{-4}, paired Student's t-test, n = 1154, 308 neurons, respectively) or remained equal in layer 5 PT neurons (**Figure 14D3**, 2nd and 3rd bin vs 2nd last and last bin, $p > 0.9$, paired Student's t-test, n = 560 neurons). Thus, in neither cell type was running onset-induced activity higher the faster the mice ran.

Similar to spontaneous turn events, also the speed of running systematically increases with learning. To elucidate whether the relationship between activity and running behavior was due to learning-related changes, I analyzed early and late sessions separately (**Figure 15**). Consistent

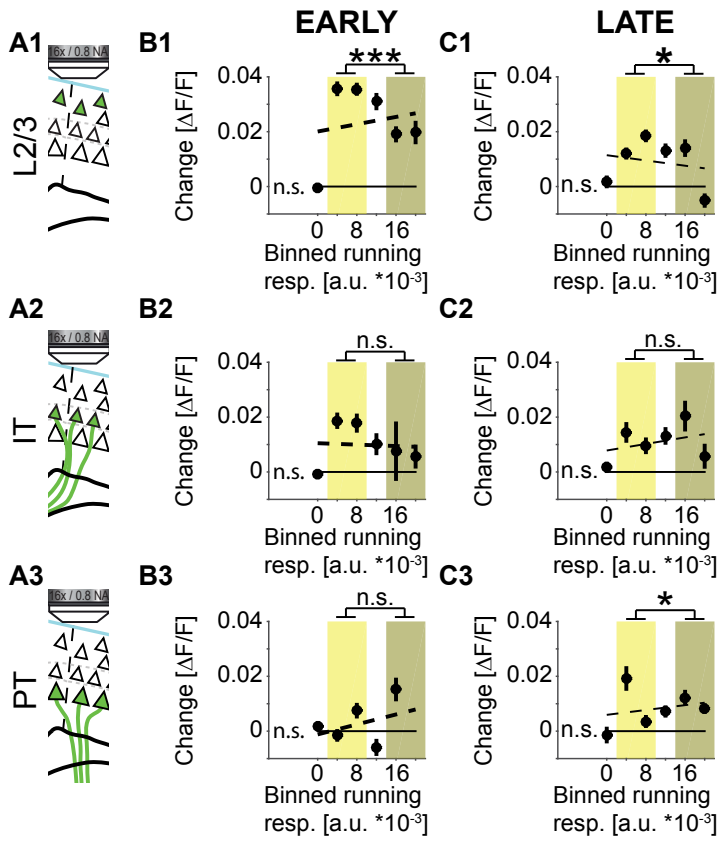


Figure 15. Relationship between running speed and running-related activity is not learning-dependent

(**A**) Schematic recording configuration for layer 2/3 (**A1**), layer 5 IT (**A2**) and layer 5 PT (**A3**). (**B**) Average activity during running onset events of different acceleration during early learning days 1-4. Error bars indicate SEM over neurons (n = 1154, 308 and 560 in layer 2/3 (**B1**), layer 5 IT (**B2**) and layer 5 PT (**B3**), respectively). Solid black line indicates 0 change [ΔF/F]. Dashed black line indicates a line fit to the data. Shading indicates bins contributing to generate average running speed and activity trace in (**B**) and (**C**). ***, $p < 0.0005$. Activity in zero bin was not significantly different from 0. (**C**) Same as in (**B**) but for late learning days 5-8.

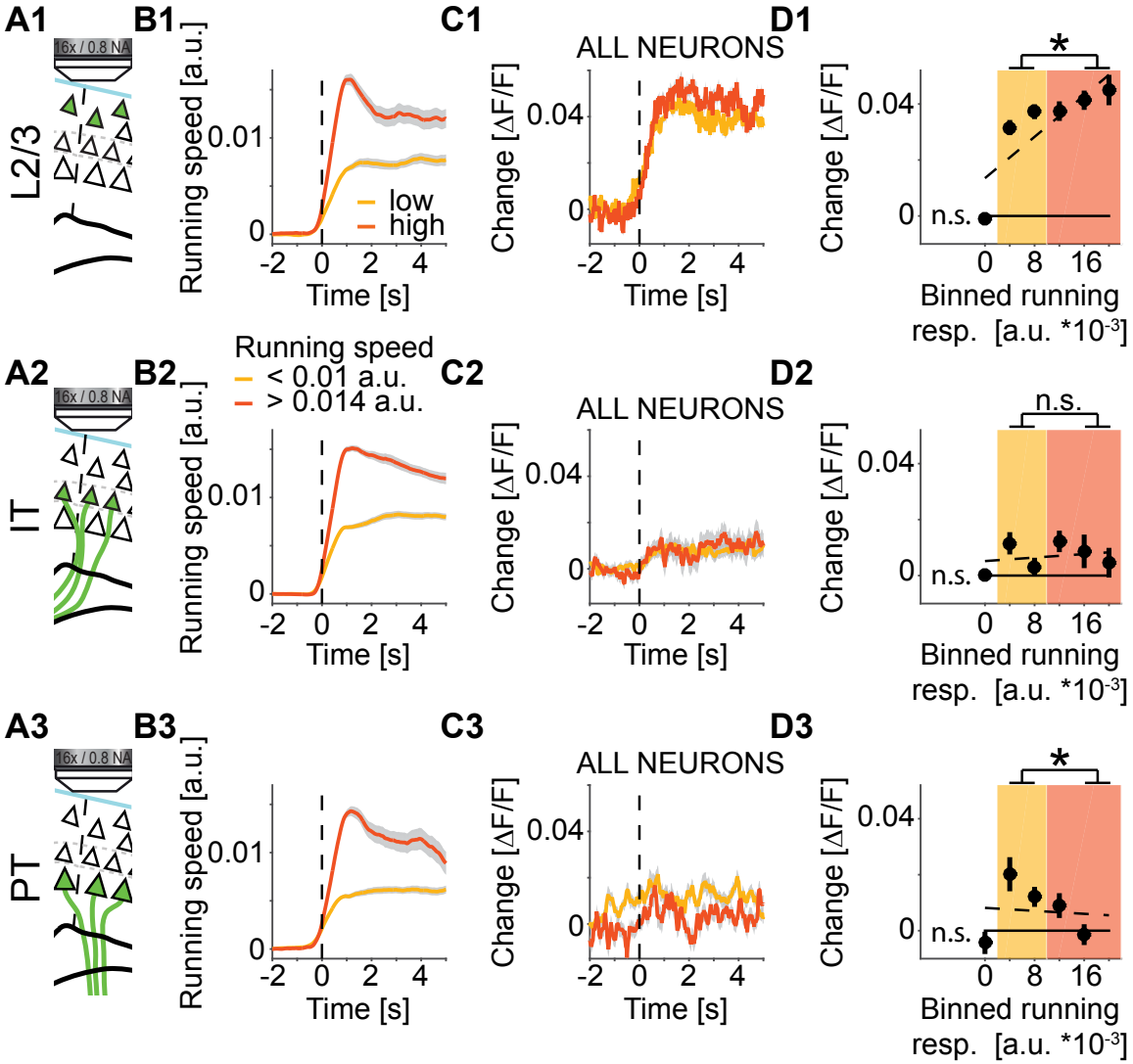


Figure 16. Activation during running onsets in darkness is different from running in VR

(**A**) Schematic recording configuration for layer 2/3 (**A1**), layer 5 IT (**A2**) and layer 5 PT (**A3**). (**B**) Average running speed during self-initiated locomotion in darkness in low (speed < 0.01 a.u., orange) and high (speed > 0.014 a.u., red) magnitude of running bins. Shading indicates SEM over trials. Time zero (dashed black line) marks running onset. (**C**) Average activity during running onsets binned as in (**B**). Shading indicates SEM over neurons (n = 1154, 308 and 560 in layer 2/3 (**C1**), layer 5 IT (**C2**) and layer 5 PT (**C3**), respectively). Time zero (dashed black line) marks running onset. (**D**) Average activity during running onset events of different speed. Error bars indicate SEM over neurons (n = 1154, 308 and 560 in layer 2/3 (**D1**), layer 5 IT (**D2**) and layer 5 PT (**D3**), respectively). Solid black line indicates 0 change [ΔF/F]. Dashed black line indicates a line fit to the data. Shading indicates bins used to generate average running speed and activity trace in (**B**) and (**C**). ***, 0.0005. Activity in zero bin was not significantly different from 0.

with the pooled data from all sessions (**Figure 14**), there was no strong linear trend between activity and running motor behavior in either early or late sessions for either of the three cell

types (**Figure 15B, C**, early: $p < 0.005$, $p > 0.9$, $p < 0.005$, (**B1 – B3**) and late: $p > 0.05$, 0.05, 0.3, linear trend analysis for n = 1154, 308, 560 neurons (**C1 – C3**), respectively). Moreover, in layer 2/3, activity decreased with increasing running speed in both early and late sessions (**Figure 15B1, C1,** early and late, $p < 10^{-5}$, 0.05, paired Student's t-test, n = 1154 neurons, respectively). Thus, the decrease in activation with increasing running speed is not due to learning-related changes.

Lastly, I examined the activation pattern when mice initiated locomotion in darkness, in the absence of visual cues. Similar to spontaneous turning events in darkness, data for running onsets was also more variable because of less recording time compared to the condition with visual cues. To reduce variability and consider more running onsets, the time mice had to be stationary was reduced to 3s for this analysis (**Figure 16**, see Experimental Procedures). Since mice could not predict water delivery while running in darkness, this change in the time mice had to be stationary is not expected to be influenced by reward predictive activity. I aligned activity as a function of running speed and found that in layer 2/3 activity linearly scales with running motor behavior and activity was higher for higher running speeds (**Figure 16D1**, 2nd and 3rd bin vs 2nd last and last bin, $p < 0.05$ and $p < 10^{-33}$, linear trend analysis for n = 1154 neurons). This was notably different compared to conditions in which running was with visual cues (**Figure 14**). There were no significant linear trends in either layer 5 IT or layer 5 PT neurons (**Figure 16 D2, D3**, $p > 0.1$, 0.6, n = 308 and 560 neurons, respectively) and only in layer 5 PT neurons was the relationship between activity and running behavior in darkness reminiscent to those observed in VR conditions (**Figure 16 D3,** 2nd and 3rd bin vs 2nd last and last bin, $p < 0.05$, n = 560 neurons). Activity was noisy for layer 5 recordings which obscures interpretation of the data. Of note, activity in darkness was notably higher than activity in VR conditions (see next section for detailed quantification).

5.3.4 Layer-specific learning-related changes in neuronal activation

5.3.4.1 Activation of layer 2/3 and pyramidal tract neurons during spontaneous turns is learning-dependent

Motor learning systematically alters activity in motor cortical areas. Activity correlates with and changes in a directional manner for task-related variables (Huber et al., 2012; Komiyama et al., 2010) and the prediction accuracy of behavior from recorded activity becomes better in deep layer neurons (Masamizu et al., 2014). Activity in deep layer PT neurons was shown to be biased to contralateral movements and presumably drives behavior (Li et al., 2015a) but how this bias arises is currently unknown.

I took advantage of the chronic activity imaging approach and analyzed activity of the

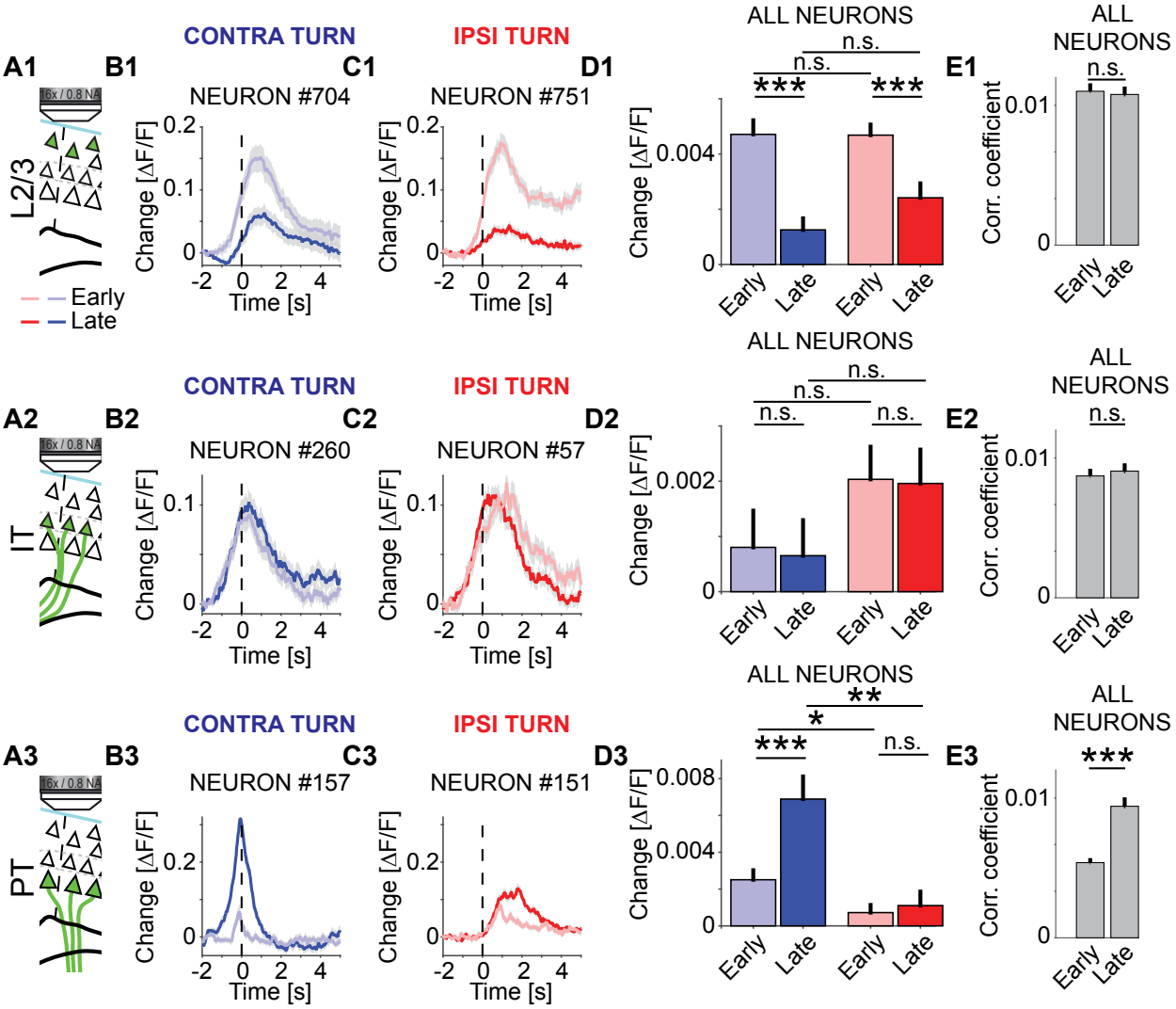


Figure 17. Learning-related, layer-specific changes in activation during spontaneous turns

(**A**) Schematic recording configuration for layer 2/3 (**A1**), layer 5 IT (**A2**) and layer 5 PT (**A3**). (**B**) Example cell average response during contraversive spontaneous turning early (light blue) or late (dark blue) in learning. Time zero marks onset of turn. (**C**) Same as (**B**) but for ipsiversive turns. (**D**) Average population response of all cells at different times in learning to either contraversive (blue) or ipsiversive (red) spontaneous turns. Error bars are SEM over neurons (n = 1154, 308, 560 in layer 2/3 (**D1**), layer 5 IT (**D2**) and layer 5 PT (**D3**), respectively). *, **, ***, $p < 0.05$, 0.005, 0.0005. (**E**) Average Pearson's correlation coefficient of activity and turning behavior. Error bars are SEM over neurons (n = 1154, 308, 560 in layer 2/3 (**E1**), layer 5 IT (**E2**) and layer 5 PT (**E3**), respectively). ***, $p < 0.0005$.

same neurons during early and late stages of learning when mice were spontaneously initiating turning events. With learning, activity in layer 2/3 neurons during both contraversive and ipsiversive turns decreased (**Figure 17D1**, contra- and ipsiversive turns, early vs late, $p < 10^{-9}$ and $p < 10^{-4}$, respectively, paired Student's t-test, n = 1154 neurons) and was not biased to either

turning direction at either learning stage (**Figure 17D1**, contraversive early vs ipsiversive early, $p > 0.9$, contraversive late vs ipsiversive late, $p > 0.1$, paired Student's t-test, n = 1154 cells). Activity in layer 5 PT neurons with learning selectively increased only for contraversive turns while activity during ipsiversive turns remained unchanged (**Figure 17D3**, contraversive early vs late, $p < 10^{-6}$, ipsiversive early vs late, $p > 0.4$, paired Student's t-test, n = 560 neurons). Activity in layer 5 PT neurons was biased to contraversive turns already at early time points and this bias further increased with learning due to the selective increase in contraversive turn activity (**Figure 17D3,** early contraversive vs ipsiversive, $p < 0.05$, late contraversive vs late ipsiversive, $p < 0.005$, paired Student's t-test, n = 560 neurons). Activity in layer 5 IT neurons was not biased and remained unchanged by learning (**Figure 17D2**, early vs late, contraversive and ipsiversive, $p > 0.8$, contraversive vs ipsiversive early and late, $p > 0.1$, paired Student's t-test, n = 308 neurons). These results suggest that during learning of a motor task, activation in more associative superficial layer 2/3 is transferred to presumably motor-output mediating layer 5 PT. Moreover, it suggests that the bias in layer 5 PT neurons observed previously (Li et al., 2015a) is established as a result of learning. I then quantified changes in reliability of responses. I computed Pearson's correlation between neural activity and turning velocity and found an increase only in layer 5 PT neurons (**Figure 17E,** early vs late**,** $p > 0.7$, 0.7 and $p < 10^{-17}$, paired Student's t-test, n = 1154, 308 and 560 neurons (**E1 – E3**), respectively).

In summary, the spontaneous turn learning analysis showed that activation during spontaneous turning events is experience-dependent in layer 2/3 and layer 5 PT cell types and that with learning, the correlation of neuronal activity and turning behavior increases in layer 5 PT neurons.

5.3.4.2 Activation during spontaneous turns is higher in the presence of visual cues than in darkness

In previous sections I showed that activation during spontaneous turns linearly scales with the vigor of motor behavior. Turning speed also systematically changed with learning (**Figure 5F**) Learning-related differences in activation might thus be due to systematic changes in turning behavior. Additionally, average turning speed differed for turning in VR and turning in darkness which obscures direct comparison of activation amplitudes.

To account for this concern, I selected a subset of spontaneous turning events such that the speed profiles for the 4 different conditions, spontaneous turning in VR early or late, and spontaneous turning in darkness, early or late, would match (**Figure 18B,** see Experimental Procedures). Note that due to fewer events, traces for onsets in darkness were noisier. For these events, I then computed the average turning-related activity for contraversive turns in contraversive cells and ipsiversive turns for ipsiversive cells (same as **Figure 11**) under both conditions, VR and

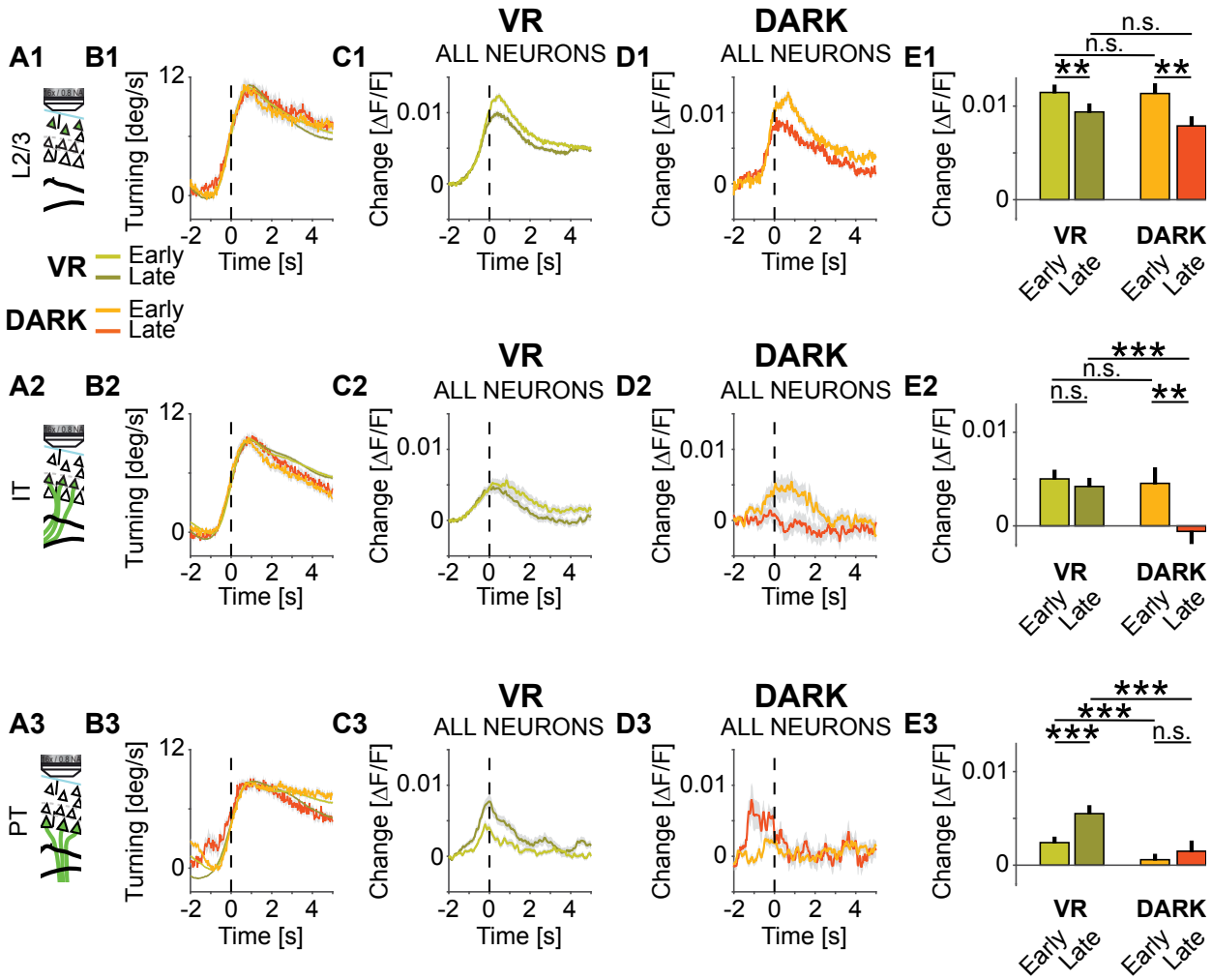


Figure 18. Activation during spontaneous turns in layer 5 is higher in the presence of visual cues than in darkness

(**A**) Schematic recording configuration for layer 2/3 (**A1**), layer 5 IT (**A2**) and layer 5 PT (**A3**). (**B**) Average turning speed matched (see Experimental Procedures) across different conditions and learning stages, VR early (pale yellow), VR late (dark yellow), DARK early (orange) and DARK late (red). Shading indicates SEM over trials. (**C**) Average activity of all cells during spontaneous turning in VR of the traces selected in (**B**). (**D**) Same as (**C**) but for DARK condition. (**E**) Average response to spontaneous turns selected in (**B**) for either of the four conditions. Activation largely paralleled the pattern reported previously (**Figure 17**). Error bars are SEM over neurons (n = 1154, 308, 560 in layer 2/3 (**E1**), layer 5 IT (**E2**) and layer 5 PT (**E3**), respectively). **,***, $p < 0.005, 0.0005$.

darkness (**Figure 18C, D**). I then compared these responses and found that responses tended to be higher in deep layers when mice were initiating spontaneous turns in the presence of visual cues as compared to turning in darkness. In detail, activation during spontaneous turns in VR decreased in layer 2/3 and increased in layer 5 PT neurons but remained unchanged in layer 5 IT

(**Figure 18E,** VR early vs late, $p < 0.005$, 10^{-8} for layer 2/3 and layer 5 PT (n = 1154, 560 neurons) and $p > 0.2$ for layer 5 IT (n = 308 neurons), paired Student's t-test). This is consistent with the learning-related changes observed previously. In darkness, activation for layer 2/3 and layer 5 IT decreased but remained unchanged for layer 5 PT neurons (**Figure 18E**, DARK early vs late, $p < 0.005$, 0.005, $p > 0.2$ for layer 2/3, layer 5 IT and PT, paired Student's t-test, n = 1154, 308 and 560 neurons, respectively). This suggests that activation during initialization of a spontaneous turn in layer 5 is of different dynamics in VR and darkness conditions. These findings are consistent with the idea of a predictive coding circuit design in which activity during VR motor behavior could subserve to update internal models but would not be useful while navigating in darkness and is thus reduced in a learning-dependent manner.

Lastly, I compared responses in VR with those during darkness (**Figure 18E**). Activation in VR, if not already higher at the early stage of learning, did become so by the late stage of learning for layer 5 cell types (**Figure 18E2 – E3**, VR early vs DARK early, $p > 0.7$ and $p < 0.0005$, and VR late vs DARK late, $p < 0.0005$ and 0.0005 paired Student's t-test, n = 308, 560 cells, in layer 5 IT and PT, respectively). Dynamics in layer 2/3 was of equal direction and magnitude such that learning did not have an effect on the difference on turning-related activity in VR compared to darkness (**Figure 18E1**, VR early vs DARK early, $p > 0.8$ and VR late vs DARK late, $p > 0.05$, paired Student's t-test, n = 1154 cells). These results suggest that spontaneously initiating turning in VR engages deep layer motor cortex more than turning in darkness, despite identical motor behavior.

In summary I find distinct learning-related dynamics in superficial layer 2/3 and layer 5 IT and PT neurons which are accompanied in deep layers by a higher activation observed during turning in VR compared to turning in darkness. This might be explained in a predictive coding framework of motor cortical function (see Discussion).

5.3.4.3 Activation of layer 2/3 and pyramidal tract cells during running onsets is learning-dependent

Next, I examined activity for speed-matched running onset events during either running onset in VR or in darkness (**Figure 19**, see Experimental Procedures). Responses in layer 5 PT neurons became very noisy due to the subselection procedure. However, despite identical motor behavior, running onset responses in layer 2/3 neurons significantly decreased with learning and, at the same time increased in layer 5 PT neurons while remaining unchanged in layer 5 IT neurons (**Figure 19E,** VR early vs VR late, $p < 10^{-18}$, $p > 0.1$ and $p < 0.005$, in layer 2/3, layer 5 IT and PT, respectively, unpaired Student's t-test). In contrast to learning-related dynamics in darkness during spontaneous turning events (**Figure 18**), learning-related activation during running onsets in darkness did not significantly change in either cell type (**Figure 19E,** DARK early vs late, $p > 0.4$, 0.5 and 0.2, for layer 2/3, layer 5 IT and PT, unpaired Student's t-test). However, there was

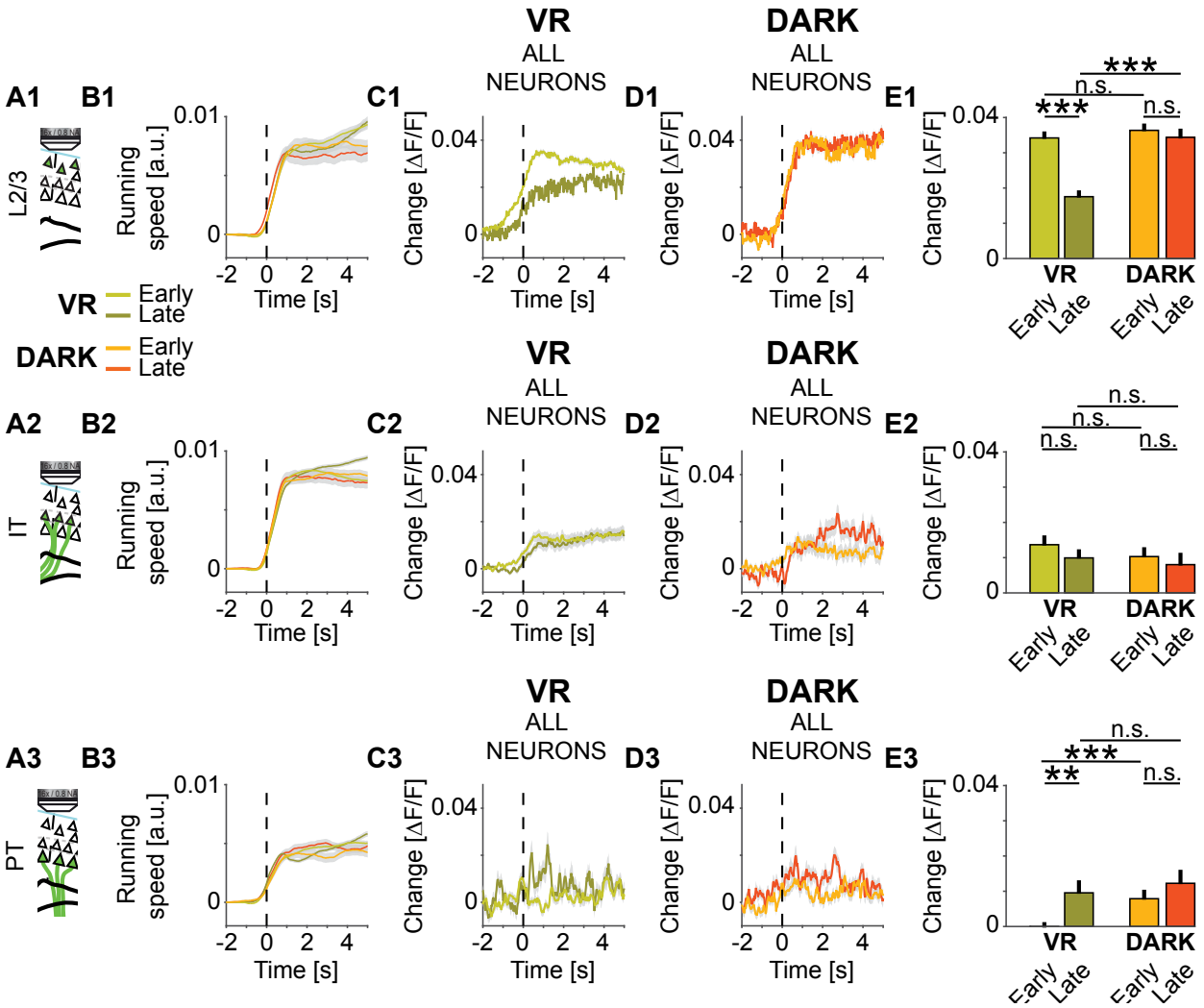


Figure 19. Learning-related changes in activation of layer 2/3 and pyramidal tract cells during running onsets

(**A**) Schematic recording configuration for layer 2/3 (**A1**), layer 5 IT (**A2**) and layer 5 PT (**A3**). (**B**) Average running onset speed matched (see Experimental Procedures) across different conditions and learning stages, VR early (pale yellow), VR late (dark yellow), DARK early (orange) and DARK late (red). Shading indicates SEM over trials. (**C**) Average activity of all cells during running onsets in VR of the onsets selected in (**B**). (**D**) Same as (**C**) but for DARK condition. (**E**) Average response to running onsets selected in (**B**) for either of the four conditions. Error bars are SEM over cells (n = 1154, 308, 560 in layer 2/3 (**E1**), layer 5 IT (**E2**) and layer 5 PT (**E3**), respectively). **,***, $p < 0.005, 0.0005$.

significantly higher, learning-dependent activation during running onsets in darkness in layer 2/3 (**Figure 19E1,** VR early vs DARK early, $p > 0.2$, VR late vs DARK late, $p < 10^{-11}$ for layer 2/3, unpaired Student's t-test). In layer 5 PT neurons, running onset-related activity was initially higher in darkness than in VR but equalized at later learning stages (**Figure 19E3**, VR early vs DARK early,

$p < 0.0005$, VR late vs DARK late, $p > 0.5$ for layer 5 PT neurons, unpaired Student's t-test) while either measure remained unchanged in layer 5 IT neurons (**Figure 19E2**, VR early vs DARK early, $p > 0.2$, VR late vs DARK late, $p > 0.5$ for layer 5 IT neurons, unpaired Student's t-test). Thus, similar to spontaneous turning events, activation during running onsets decreased in layer 2/3 but increased in layer 5 PT neurons. However, while during spontaneous turns activation in darkness was learning-dependent in layer 2/3 and layer 5 IT, this was not the case for running onsets.

Interestingly, it seemed as if much of the decrease in layer 2/3 activation could be explained by a learning-dependent elimination of preparatory activity (**Figure 19C1**, see section on preparatory activity for a more detailed account). Preparatory activity, by definition, occurs in the absence of movement. Thus, the actual movement-related activity in layer 2/3 neurons during running onsets in VR is presumably much lower, possibly in the range of the amount obsreved in the late learning stage.

5.3.5 Activation during visual offset perturbation induced turns

Motor cortical areas have frequently been probed in the context of stimulus-induced motor behaviors for example by prompting primates to execute reaches in instructed-delay tasks (Churchland et al., 2012; Kaufman et al., 2014) or forced-choice licking in mice in response to somatosensory stimuli (Huber et al., 2012; Li et al., 2015a). These tasks usually require extensive training and the advantage lies in comparing baseline activity during quiescence and, depending on task structure, movement preparation to movement-related activity during active behavior during task performance. With the present task, my question was to investigate activity during two identical turning behaviors with respect to whether the execution of the behavior was spontaneous (see previous section) or forced as during the visual offset perturbations (**Figure 5E - I**) which occurred pseudo-randomly. As a result, spontaneous turning could be pre-planned and compensated for by the mice but turning in response to visual offsets was unpredictable and could reveal abrupt changes in motor plans.

5.3.5.1 Stimulus-driven activity in layer 2/3 and layer 5 intratelencephalic neurons

I took advantage of visual offset perturbations and aligned both turning behavior and activity to the stimulus onset (**Figure 20**). Since the precise trajectory by which mice navigated through the virtual tunnel was not strictly reinforced and visual offset perturbations were unpredictable to the mice both in time and direction, this resulted in events in which offsets occurred either in favor of the mice's heading direction, shifting the reward zone more towards the center of the

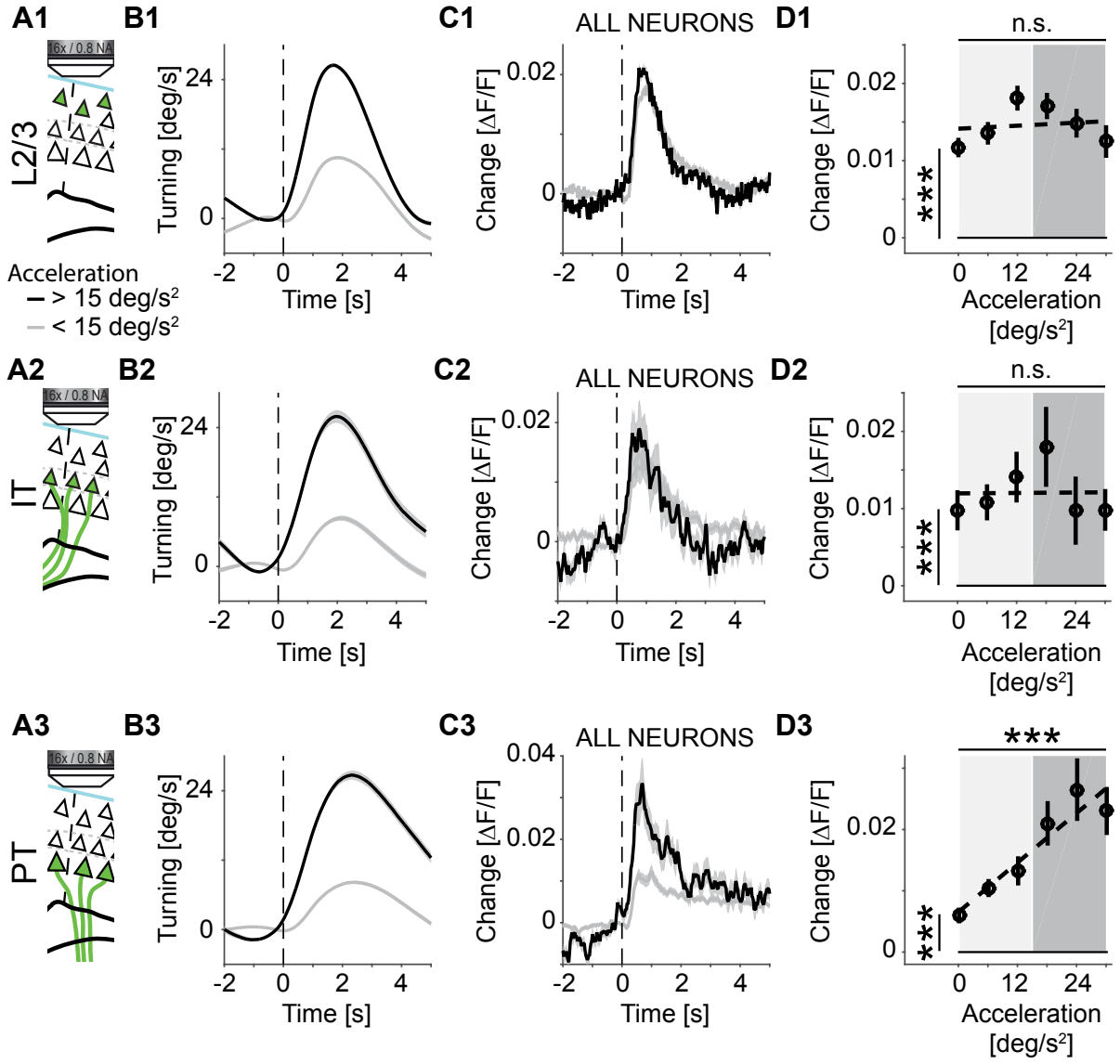


Figure 20. Stimulus-driven activation in layer 2/3 during visual offset induced corrective turns

(**A**) Schematic recording configuration for layer 2/3 (**A1**), layer 5 IT (**A2**) and layer 5 PT (**A3**). (**B**) Average induced turning speed in low (acceleration < 15 deg/s^2, grey) and high (acceleration > 15 deg/s^2, black) magnitude of turning bins. Shading indicates SEM over trials. Time zero (dashed black line) marks onset of visual offset. (**C**) Average activity during induced turns binned as in (**B**). Shading indicates SEM over neurons (n = 1154, 308 and 560 in layer 2/3 (**C1**), layer 5 IT (**C2**) and layer 5 PT (**C3**), respectively). Time zero (dashed black line) marks onset of visual offset. (**D**) Average activity during induced turning events of different acceleration. Error bars indicate SEM over neurons (n = 1154, 308 and 560 in layer 2/3 (**D1**), layer 5 IT (**D2**) and layer 5 PT (**D3**), respectively). Solid black line indicates 0 change [ΔF/F]. Dashed black line indicates a line fit to the data. Grey shading indicates bins used to generate average turning speed and activity trace in (**B**) and (**C**). ***, $p < 0.0005$.

field of view (as if mice were running straight) such that less vigorous turning was required or in events which were disadvantageous to the mice, shifting the reward zone further away from the center, requiring more corrective turning.

In all three data sets, I pooled left and right induced corrective turns and assigned bins as a function of turning speed (**Figure 20B**). Importantly, these bins were identical to the turning speed bins used for the spontaneous turn analysis (see **Figure 11**). I then selected cells based on their response to spontaneous turn events and computed the mean response of contraversive cells to contraversive induced turns and ipsiversive cells to ipsiversive induced turns (**Figure 20C**). In contrast to activation during spontaneous turns, activation during induced turns in neither layer 2/3 nor layer 5 IT neurons linearly scaled with corrective turning motor behavior (**Figure 20D1, D2**, first vs last bin, $p > 0.6, 0.1$, paired Student's t-test for n = 1154, 308 neurons in layer 2/3, layer 5 IT, respectively). Examination of example cells revealed similar trends as observed on the population level (**Figure S3A, B**). Regression analysis did not reveal statistically significant linear trends (**Figure 20D1, D2,** $p > 0.4, 0.8$, linear trend analysis in layer 2/3, layer 5 IT). Thus, activation for low and high speed turning was indistinguishable in layer 2/3 and layer 5 IT neurons suggesting different coding schemes for spontaneous and induced turning behavior.

I then examined the neuronal activation when mice were not changing their motor program. Interestingly, in the first bin, when there was no detectable change in ongoing motor behavior, activation was significantly greater than zero in both layer 2/3 and layer 5 IT neurons (**Figure 20D1, D2**, first bin vs zero change [$\Delta F/F$], $p < 10^{-30}$, 10^{-5}, Student's t-test for zero mean in layer 2/3 (n = 1154 neurons) and layer 5 IT (n = 308 neurons)). Together with the observation that activation in layer 2/3 and layer 5 IT neurons also did not change depending on the turning speed of the mice, this implies that most, if not all, of the observed activation is stimulus-driven and independent of the subsequent motor behavior.

5.3.5.2 Activity linearly scales with mouse behavior in pyramidal tract neurons

Next I investigated activation of layer 5 PT neurons during induced turns as a function of acceleration (**Figure 20A3 - D3**). In contrast to layer 2/3 and layer 5 IT neurons but similar to spontaneous turns, activation in layer 5 PT neurons was linearly dependent on subsequent turning motor behavior: The higher the turning speed of the mice, the more activity was observed (**Figure 20D3**, first vs last bin, $p < 0.0005$, paired Student's t-test, n= 560 neurons, $p < 10^{-7}$, linear trend analysis). I then examined activation of layer 5 PT neurons in the first speed bin. In the absence of corrective turning behavior, similar to layer 2/3 and layer 5 IT neurons, activation in layer 5 PT neurons was significantly different from zero (**Figure 20D3**, $p < 10^{-9}$, Student's t-test for zero mean). Thus, layer

5 PT neurons were also partly stimulus-driven but a large fraction of the observed activity was still dependent on the subsequent turning speed.

5.3.6 Spontaneous and induced turns activate the same cells

The results for spontaneous and induced turning events suggest distinct activity dynamics in layer 2/3 depending on the presence of a stimulus which drives motor behavior. Activity patterns during spontaneous and induced turns could be different because a dedicated subset of neurons is active during either visual offset perturbation and during spontaneous turns or the same neurons become active differentially for the two turn types.

In a first level of analysis, I examined responses of individual neurons during either contra- or ipsiversive spontaneous or induced turns (**Figure 21**). Spontaneous turn responses in all layers were anti-correlated (**Figure 21B**): Neurons which were active when mice were turning in one direction were likely to decrease their activity when mice initiated turns in the opposite direction. Example neuronal responses illustrate these patterns (**Figure S4,** spontaneous turns). Next, I analyzed the same neurons for their induced turn responses (**Figure 21C**) and color-coded them according to their response selectivity during spontaneous turns. If during induced turns, the only determinant of a neuron's response would be the direction of turning, the two analyses

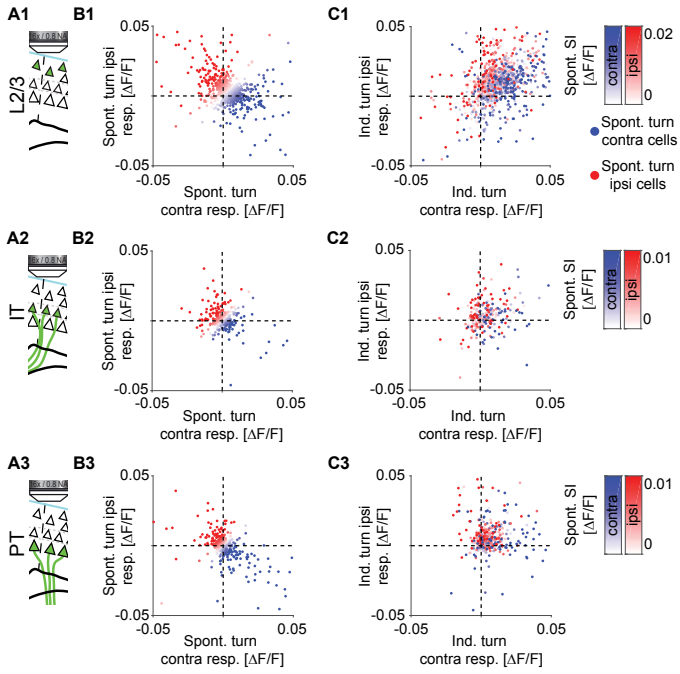


Figure 21. Layer 2/3 neurons are active regardless of corrective turning direction

(**A**) Schematic recording configuration for layer 2/3 (**A1**), layer 5 IT (**A2**) and layer 5 PT (**A3**). (**B**) Contra- and ipsiversive spontaneous turn responses of individual neurons (n = 1154, 308 and 560 in layer 2/3 (**B1**), layer 5 IT (**B2**) and layer 5 PT (**B3**), respectively). neurons were color-coded according to their selectivity during spontaneous turns (see Experimental Procedures). Dashed lines indicate 0 [ΔF/F].(**C**) Same as (**B**) but for induced turns. Note many layer 2/3 data points are located inside the first quadrant.

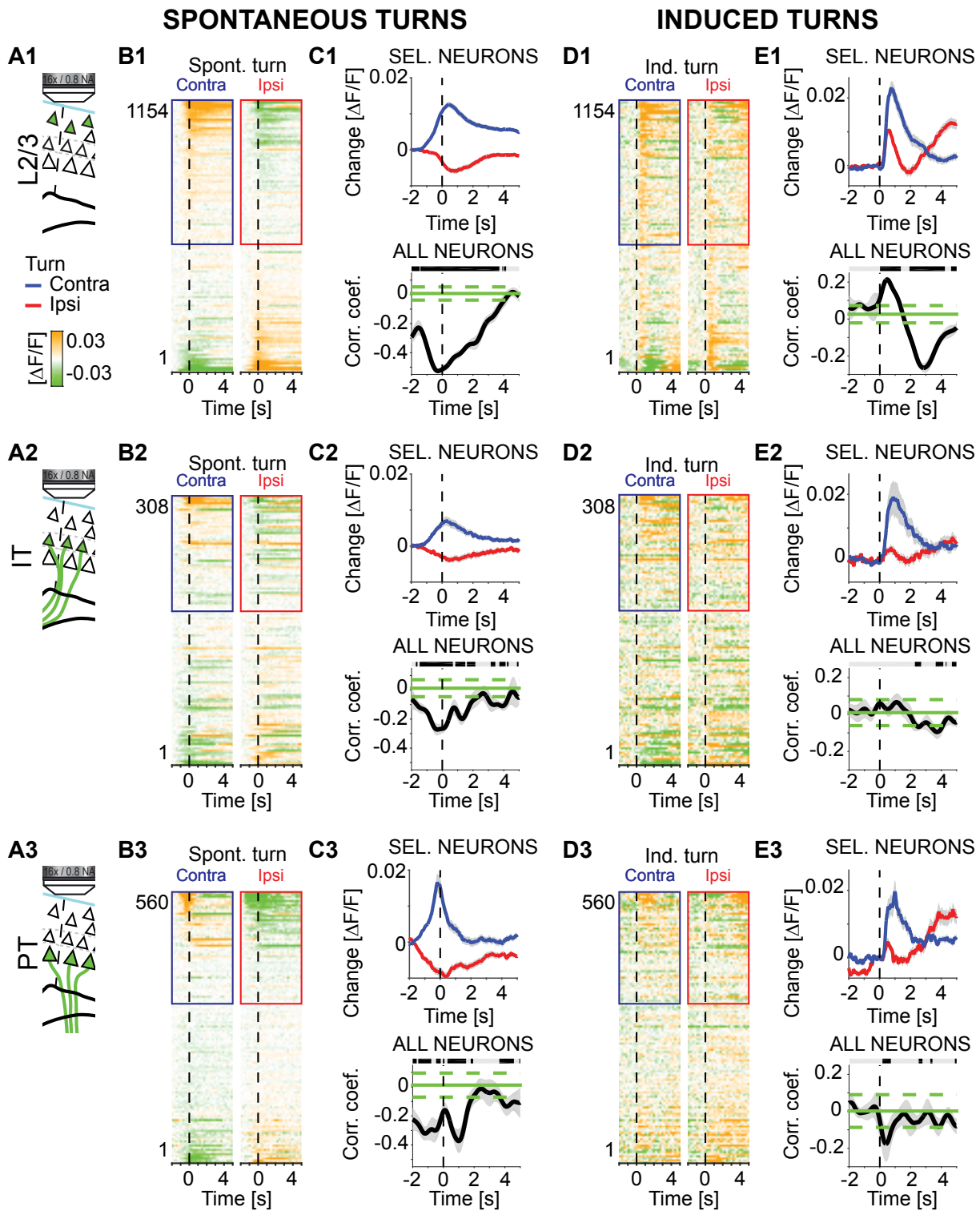


Figure 22. Co-activation of layer 2/3 during induced turns

(**A**) Schematic recording configuration for layer 2/3 (**A1**), layer 5 IT (**A2**) and layer 5 PT (**A3**). (**B**) Individual neuron's average activity to either contra- (left panel) or ipsiversive (right panel) spontaneous turns sorted to selectivity during spontaneous turns. Time zero (dashed black line) marks turn onset. Number of neurons indicated to the left of heatplot. Boxes mark contraversive turn. *Legend continued on next page*

cells (see Experimental Procedures). (**C**) Upper panel: Average activity of contraversive turn neurons (boxed in (**B**)) during contra- (blue) or ipsiversive (red) spontaneous turns. Note diverging activity patterns. Shading indicates STD over trials. Time zero (black dashed line) marks turn onset. Lower panel: Pearson's correlation coefficient of population response vectors binned in time (see Experimental Procedures). Solid green line indicates correlation of population response vectors during random onsets. Dashed green line indicates STD of random onset correlation. Time zero (dashed black line) indicates turn onset. Black ticks indicate statistically significantly different ($p < 0.05$) bins of data and random onset. (**D**) Same as (**B**) but for induced turns. Time zero (dashed black line) marks onset of visual offset perturbation. Note initial activation during either induced turn for many layer 2/3 neurons. (**E**) Same as (**C**) but for induced turns. Time zero (black dashed line) marks onset of visual perturbation. Note that initially prominent positive correlation in layer 2/3 which was absent in layer 5 data.

for spontaneous and induced turns should look the same. Instead, I find that a large fraction of neurons is located in the first quadrant suggesting that irrespective of the turning direction, responses were positive (**Figure 21C**). This effect was most prominent in layer 2/3 and was in contrast to the spontaneous turns (**Figure 21B**) in which only a minority of neurons showed this pattern of activity. I then looked at the distribution of neurons depending on their spontaneous turn response. Neurons with higher spontaneous turn selectivity (SI, see Experimental Procedures) were assigned a darker hue (**Figure 21**). However, there was no clear pattern of those cells when assessed in induced turn conditions: Neurons which had a high selectivity to a spontaneous turn direction could have a low induced turn response and vice versa. Importantly, the majority of spontaneous turn neurons was also active during the induced turn in the same direction. In summary, spontaneous and induced turn behavior employ similar neuronal circuits and in layer 2/3, neurons were co-active during both induced turning directions.

Next, I analyzed the time course of responses around the time when mice spontaneously initiated turns and compared that to the turns induced by visual offset perturbations (**Figure 22**). For all neurons, activity was aligned to either contra- or ipsiversive spontaneous turn events (**Figure 22B**, same sorting for contra- and ipsiversive turns). Activity for these two turns was anti-correlated. I selected contraversive turn neurons (**Figure 22B,** boxed) and averaged their activity to either contra- or ipsiversive turns (**Figure 22C,** upper panel, blue: contraversive turns, red: ipsiversive turns) and found a diverging activity pattern. This shows that on a population level, activation patterns for spontaneous turns were anti-correlated, in all cell types. To directly test this hypothesis, I computed the correlation coefficient of the neuronal responses vectors for all neurons for contra- and ipsiversive turns at different time bins around the turn onset (**Figure 22C,** lower panel, see Experimental Procedures). Shortly after initiation of spontaneous turns, the correlation was strongly negative (**Figure 22C,** lower panel, aligned (black) vs random (green) at $t = 0.25s$, $p < 0.0005$, 0.005 and 0.005, paired Student's t-test, in layer 2/3, layer 5 IT and PT,

n = 1154, 308 and 560 neurons, respectively) see Experimental Procedures for generation of randomized traces) but approached zero at the end of the turn. This provides support for the notion that during spontaneous turns, population activity in all cell types is anti-correlated.

I then examined the activation during visual offset induced turns in the same manner (**Figure 22D, E**). Notably, in layer 2/3, contraversive turn neurons (same as **Figure 22B, C**) were initially active regardless of the turning direction. This was markedly different from spontaneous turns where I observed activity of opposite signs. In layer 5 IT and layer 5 PT neurons, no such co-activation could be observed. Note, that spontaneous turn neurons were also active during induced turns (**Figure 22C, E,** upper panel). I further characterized this effect by computing the correlation coefficient of the neuronal response vectors for contra- and ipsiversive turns and found that the correlation after stimulus onset was significantly positive in layer 2/3 neurons but negative in layer 5 PT neurons and did not change in layer 5 IT neurons (**Figure 22E,** lower panel, at t = + 0.25 s, data (black) vs random (green), $p < 0.0005$, $p > 0.2$, $p < 0.005$, paired Student's t-test, in layer 2/3, laser 5 IT and layer 5 PT for n = 1154, 308 and 560 neurons). Note, that in layer 2/3 neurons, the correlation flipped sign in the course of the turning events (**Figure 22E1,** intersection with zero, t = 1 s). Moreover, correlation became significantly negative (t = 2.5 s, data (black) vs random (green), $p < 0.005$, paired Student's t-test, n = 1154 neurons) suggesting similar activity patterns as those observed during spontaneous turns. Thus, in layer 2/3 the population of neurons is activated during visual offset perturbation induced turns regardless of the subsequent turning direction but later during the turn, activation became reminiscent of spontaneous turning behavior.

5.3.7 Initial co-activation of layer 2/3 during induced turns is of equal strength

The previous analysis suggested, during spontaneous and induced turns, dynamics that are different in layer 2/3 neurons but similar in layer 5 IT and PT neurons. Data suggested that neurons in layer 2/3 were initially co-active which was not the case for deep layer neurons. Next, I assessed the strength of co-activation. Neurons could become equally co-active during either induced turn or there could be a strong bias, the activity favoring already one turn direction over the other.

To directly compare strength, I thus determined how induced turn activity evolves, but in the frame of reference of spontaneous turns (**Figure 23**). For each neuron, I computed separately the activity during spontaneous contra- and ipsiversive turns (see Experimental Procedures). This high-dimensional vector (number of dimensions = number of neurons) spanned a manifold. Induced turn responses of the same neurons were then projected onto two dimensions using the spontaneous turn responses as basis vector space which I orthogonalized, centered to baseline activity and normalized to the mean population peak response during spontaneous turns. I then compared the resulting trajectories.

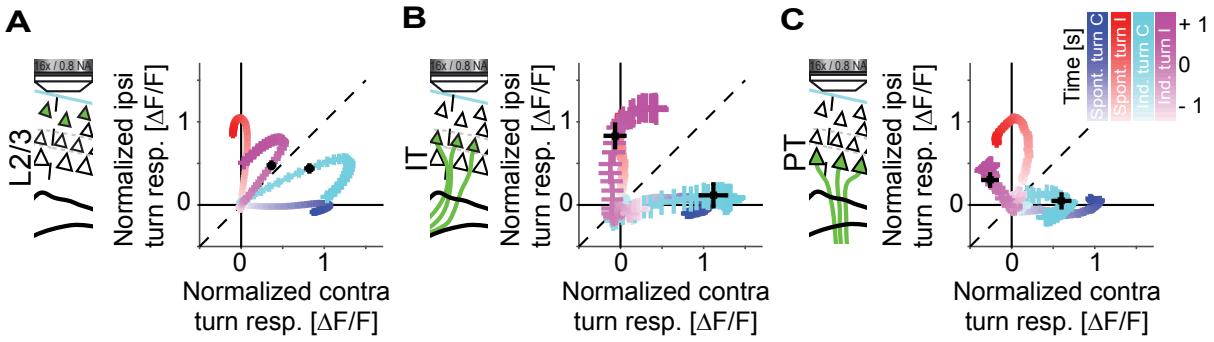


Figure 23. Induced turns in opposite directions activate layer 2/3 cells with equal strength

Population response vectors binned in time around the onset of turning events (blue: spontaneous contraversive turn, red: spontaneous ipsiversive turn) or onset of visual perturbation stimulus (cyan: induced contraversive turn, magenta: induced ipsiversive turn) and projected into the two-dimensional space spanned by spontaneous turn basis vectors in layer 2/3 (**A**), layer 5 IT (**B**) and layer 5 PT (**C**). Color hues show temporal progression as indicated. Solid black lines mark zero [ΔF/F] response. Dashed black line marks identity of responses. Error bars are SEM over responses in individual events. Black data point indicates onset of turning behavior in response to visual perturbations.

The resulting spontaneous turn trajectories evolved around the axes (**Figure 23A – C,** blue and red for contra- and ipsiversive spontaneous turns). By definition, activity during spontaneous turns thus occupied very distinct regions in this basis vector space. I then assessed the induced turn responses in the same way (**Figure 23A – C,** magenta and cyan for contra- and ipsiversive induced turns). If during induced turns, the activation for contra- and ipsiversive turns was equally strong, trajectories should evolve around the identity line.

In layer 2/3 I found that induced turn trajectories followed the identity line, suggesting that activation for contra- and ipsiversive induced turns was initially equal. After turning onset (**Figure 23A – C**, black data point), activity patterns collapsed onto spontaneous turn patterns: the contraversive induced turn pattern collapsed towards the region in the basis vector space occupied by the contraversive spontaneous turn trajectory and the ipsiversive induced turn pattern collapsed towards the region in the basis vector space occupied by the ipsiversive spontaneous turn trajectory. This indicates that, as the turn progressed, the preferred turn's activation remained high but activation to the non-preferred (opposite) direction ceased. In layer 5, initial induced turn trajectories paralleled those of spontaneous turns. This is consistent with the previous finding that in layer 2/3 neurons, the population response vectors for induced turns were initially positively correlated and, in the course of turning, became negatively correlated, providing further support for the notion that activity dynamics during induced turn are distinct in layer 2/3 and layer 5 neurons.

5.3.8. Context-dependent modulation of activity

Motor cortex activity was reported to be modulated by various features not directly implicated in movement generation such as visual stimuli (Shen and Alexander, 1997a) or reward information (Kapogiannis et al., 2008). These signals could be useful to behaviorally cope with a complex natural environment. I examined context-dependent modulation of activity in the navigation task.

5.3.8.1 Reward modulation of local motor cortical circuits

Motor cortex is an important site for various types of motor learning (Kawai et al., 2015; Komiyama et al., 2010) and dopaminergic projections have been reported to be important for motor skill learning (Hosp et al., 2011) suggesting potent mechanisms ensuring cellular reinforcement learning within local motor cortex circuitry.

To address the question if motor cortex is modulated by reward delivery, for each traversal I aligned activity to the time point of reward delivery (**Figure 24**). The only sensory modality that could provide cues for navigation and potential reward-related prediction was vision. To compare situations with and without cues, I compared activity obtained during recording in VR with those obtained during the DARK session. Moreover, reward prediction should also be dependent on experience: Mice first have to determine the task structure and the statistics of reinforcement in order to be able to make predictions. To test this idea, I split the data into early and late sessions to extract changes related to learning.

In all cell types, there was activity which ramped up as the mice approached the reward zone. Reward-anticipatory activity with learning increased in layer 5 neurons but remained unchanged in layer 2/3 neurons (**Figure 24B**, **D**, VR pre early vs late, t = - 0.5 s – 0 s, $p > 0.3$, $p < 0.005$, 10^{-16}, paired Student's t-test, in layer 2/3, layer 5 IT and PT, n = 1154, 308 and 560 neurons). This was consistent with a previous observation that dopaminergic fibers originating in the VTA primarily innervate deep layers of motor cortex (Hosp et al., 2011) and are thereby more likely to modulate deep layer 5 neurons efficiently. Concurrent with reward delivery, a grey screen was displayed to signal the time out period and motor behavior changed abruptly to licking (see **Figure 5**). Thus, activity after reward delivery likely reflects licking motor behavior. I quantified if there was a learning-dependent change in presumably licking-induced activity and how this compares to anticipatory activity prior to reward delivery. In layer 2/3 neurons, there was more activity after reward delivery than in a similar window before reward delivery indicating licking motor-related activity was present. This did not change with learning. In layer 5 IT, presumptive licking-related activity could barely be detected and this did not change in the course of learning. In layer 5 PT neurons, licking-related activity was not detectable in early sessions but emerged with learning (**Figure 24B, D,** presumed licking motor-related activity: VR pre early vs post early, $p < 10^{-17}$, $p > 0.8, 0.1$, paired Student's t-test in layer2/3, layer 5 IT and PT, n = 1154, 308 and 560

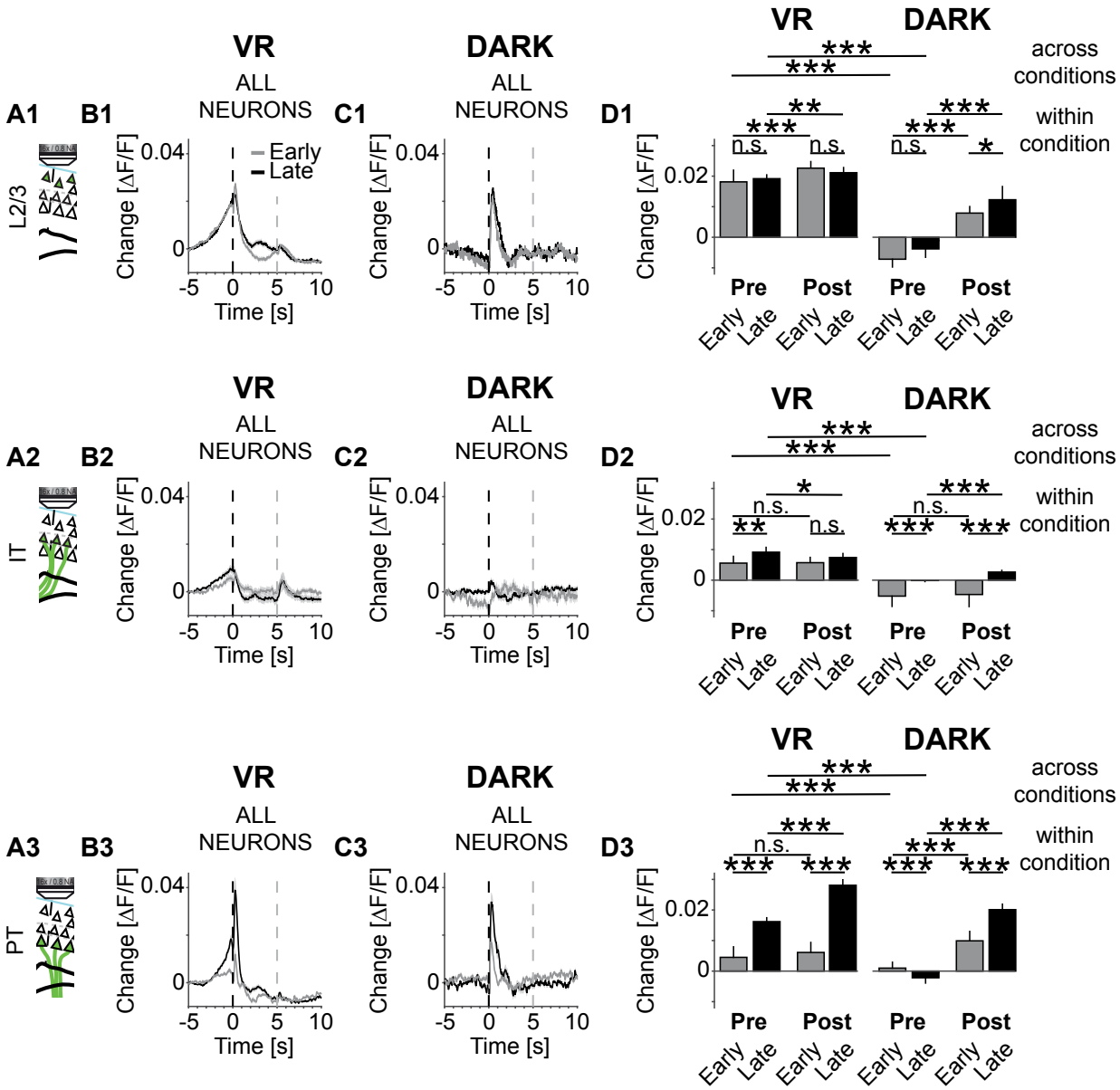


Figure 24. Experience-dependent activity during reward anticipation and lick-related motor behavior

(**A**) Schematic recording configuration for layer 2/3 (**A1**), layer 5 IT (**A2**) and layer 5 PT (**A3**). (**B**) Average population activity around the time of reward delivery (dashed black line) early (grey) or late (black) in learning in VR condition. Shading indicates SEM over neurons (n = 1154, 308 and 560 in layer 2/3 (**B1**), layer 5 IT (**B2**) and layer 5 PT (**B3**), respectively). Grey dashed line marks end of time out period. (**C**) Average population activity around the time of reward delivery (dashed black line) early (grey) or late (black) in learning in darkness. Shading indicates SEM over neurons (n = 1154, 308 and 560 in layer 2/3 (**C1**), layer 5 IT (**C2**) and layer 5 PT (**C3**), respectively). Grey dashed line marks end of time out period. (**D**) Average population response around the time of reward delivery in VR or darkness, either early or late in learning (same data shown in (**B**) and (**C**)). *, **, ***, $p < 0.05, 0.005, 0.0005$.

neurons; Learning-related increase in licking-related activity: VR post early vs late, $p > 0.2, 0.1, p < 10^{-15}$, paired Student's t-test in layer 2/3 layer 5 IT and PT, n = 1154, 308 and 560 neurons). In summary, in VR conditions, with learning activity in layer 5 PT neurons emerges which reflects both reward-anticipatory properties as well as presumably licking motor-related activity. Reward-anticipatory activity in layer 2/3 neurons was present already at early stages during learning and did not change in the course thereof.

Next, I compared activity around the reward delivery in VR with those in darkness conditions (**Figure 24C, D,** dark). If the ramping of activity in VR reflected reward-anticipatory activity, this type of activation should not be present in darkness since mice cannot predict when rewards would be delivered. Ramping activity as assessed by activity levels prior to reward delivery was not present in darkness in neither cell type. Consequently, activation prior to reward delivery was much stronger in VR than in dark conditions suggesting that the activation observed in VR reflects reward anticipation (**Figure 24D,** VR pre early vs DARK pre early, $p < 10^{-39}$, 10^{-7}, 0.0005, paired Student's t-test in layer 2/3, layer 5 IT and PT, n = 1154, 308 and 560 neurons). Even with increasing expertise in VR, running in darkness remains by definition uncued implying that mice cannot form reward predictions despite learning. As a consequence, reward anticipatory in darkness could not be observed at late stages of learning and remained significantly less compared to VR (**Figure 24D,** VR pre late vs DARK pre late, $p < 10^{-29}$, 10^{-6}, 10^{-17}, paired Student's t-test in layer 2/3, layer 5 IT and PT, n = 1154, 308 and 560 neurons).

After reward delivery, the licking motor behavior remains the same as mice still collect rewards. Lick-related activity should therefore be detectable regardless of conditions. Indeed, in darkness in layer 2/3 and layer 5 PT neurons, presumably licking-related activity was still present, higher than reward anticipatory activity throughout learning but also modulated by learning (**Figure 24D,** DARK pre early vs DARK post early, $p < 10^{-101}$, $p > 0.4$, $p < 10^{-9}$, DARK pre late vs post late, $p < 10^{-83}$, 0.0005, 10^{-14}, DARK post early vs late (lick-related activity learning), $p < 0.05$, 10^{-5}, 10^{-5}, paired Student's t-test in layer 2/3, layer 5 IT and PT, n = 1154, 308 and 560 neurons, respectively).

In summary, ramping activity levels in VR conditions in all cell types likely reflect reward-anticipatory activity. Licking motor behavior related activity is unaffected by VR or darkness conditions.

5.3.8.2 Unique activation patterns of layer 5 intratelencephalic projection neurons

Positive reinforcement was useful to train mice to expert performance in the task. The blue reward zone clearly constituted a cue capitalized on by the mice to efficiently navigate. However,

I intentionally introduced two more cues, the texture change and the time out period which could help delineating task and trial structure and could therefore be useful during learning.

5.3.8.2.1 Layer 5 intratelencephalic projection neurons are transiently active when the texture changes

I first explored whether or not there was activation of motor cortex at the time points when the texture changes. In the standard paradigm, textures always changed exactly halfway through the tunnel (see Experimental Procedures). In early sessions, activity in layer 2/3 and layer 5 PT neurons rose as the mice passed by the texture change but this rise was initially not significant in layer 5 IT neurons (**Figure 25B, C**, early vs random, $p < 10^{-7}$, $p > 0.1$, $p < 0.05$, Student's t-test for zero mean, layer 2/3, layer 5 IT and PT in n = 1154, 308 and 560 neurons). In the course of learning, as mice passed by the texture change, activation did not increase in layer 2/3 but strongly increased in layer 5 IT and PT neurons (**Figure 25B, C,** early vs late, $p > 0.2$, $p < 10^{-7}$, 10^{-8}, paired Student's t-test and late vs random, $p < 10^{-10}$, 10^{-9}, 10^{-34}, Student's t-test for zero mean, in layer 2/3, layer 5 IT and PT in n = 1154, 308 and 560 neurons, respectively). Note, that activation of layer 5 IT neurons was transient and decayed after mice passed the texture change suggesting a signal specific to the texture change. Activity in layer 2/3 and layer 5 PT neurons was consistent with the ramp up of activity in anticipation of the reward delivery observed previously. Thus, in this analysis, layer 5 IT neurons show a unique pattern of activation when mice passed by the texture change.

To more thoroughly elucidate the phenotype in layer 5 IT neurons, in a subset of traversals, I omitted the texture change and instead displayed either texture A or B throughout the tunnel (P = 0.1, randomly interleaved, see Experimental Procedures). If layer 5 IT neurons specificly signaled the texture change, activity should not change when mice pass by at the midpoint of a single-textured tunnel. Event numbers were matched to account for different probabilities of either dual- or single-textured traversals. Single neurons were more active at the midpoint of the tunnel when textures changed (**Figure 26B, C**). On the population level, transient activity of the top 50% of neurons was significantly higher in traversals containing a texture change compared to single-textured traversals (**Figure 26D, E**, A-B vs A-A and B-B, $p < 10^{-5}$ and $p < 10^{-5}$, A-A vs A-B, $p > 0.9$, paired Student's t-test, top 50% of neurons, n = 154 neurons, see Experimental Procedures for cell selection). This provides support for the notion that transient activity in layer 5 IT neurons at the midpoint of the tunnel is driven by the change in tunnel wall texturing.

5.3.8.2.2 Intratelencephalic projection neurons are active at visual cue onset

Layer 5 IT neurons could not only be driven by the texture change but activation might generalize to cues in the environment which allow the mice to predict the task and trial structure. Another

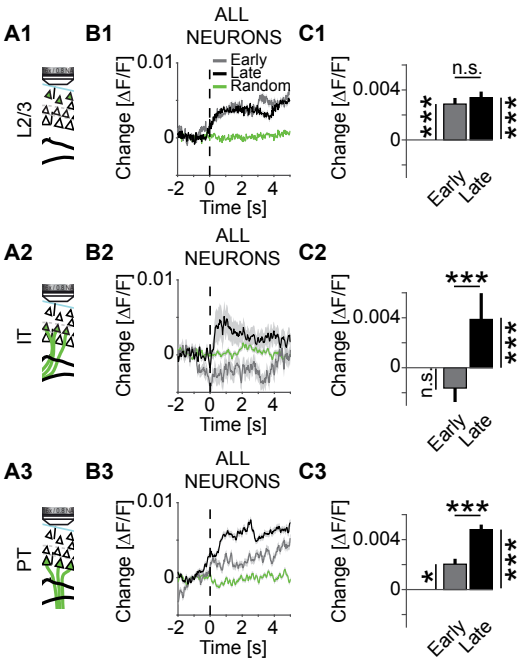


Figure 25. Learning-related activation during texture changes

(**A**) Schematic recording configuration for layer 2/3 (**A1**), layer 5 IT (**A2**) and layer 5 PT (**A3**). (**B**) Average population activity aligned to the onset of the texture change in the virtual tunnel early (grey) or late (black) in learning. Shading indicates SEM over neurons (n = 1154, 308 and 560 cells, in layer 2/3 (**B1**), layer 5 IT (**B2**) and layer 5 PT (**B3**)). Green line indicates average of random onsets. Time zero (dashed black line) marks texture change. (**C**) Average population response to texture change early or late in learning. Error bar indicates SEM over neurons (n = 1154, 308 and 560 cells, in layer 2/3 (**C1**), layer 5 IT (**C2**) and layer 5 PT (**C3**)). *, ***, p < 0.05, 0.0005.

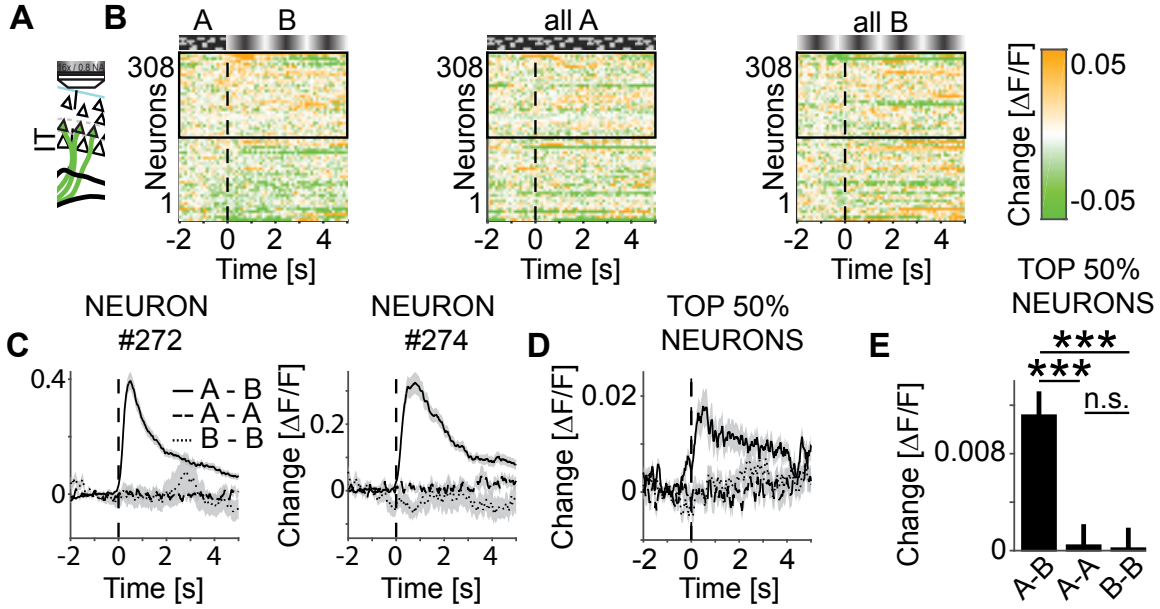


Figure 26. A subset of layer 5 IT neurons is transiently active during texture changes

(**A**) Schematic recording configuration in layer 5 IT neurons. (**B**) Average activity of individual layer 5 IT cells during traversals with texture change (left panel) or either single-textured (middle and right panel) traversals. Time zero marks midpoint of tunnel throughout figure. Box indicates top 50% of neurons. (**C**) Average example neuronal activity during passing of tunnel midpoint with (solid) or without texture change (dashed or dotted). Shading indicates SEM over trials. (**D**) Average activity of top 50% of neurons during passing of tunnel midpoint with (solid) or without texture change (dashed or dotted). Shading indicates SEM over neurons (n = 154). **E**) Average response of top 50% of neurons to different tunnel texture conditions. Error bars indicates SEM over neurons (n = 154). ***, p < 0.0005.

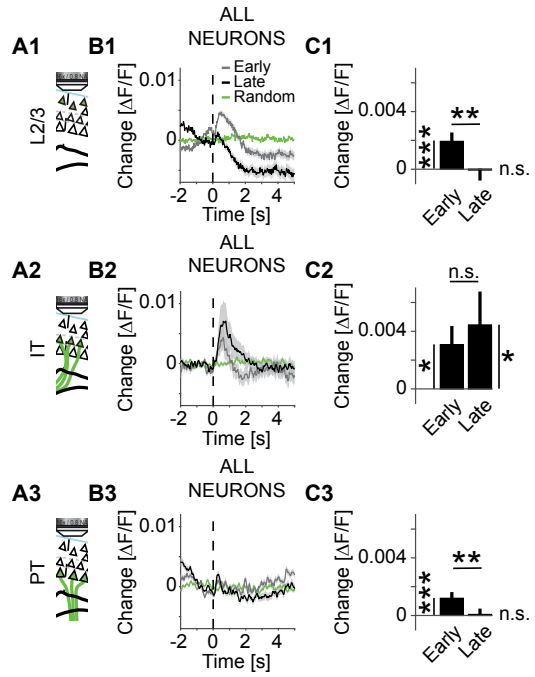


Figure 27. Activation during onset of visual cues

(**A**) Schematic recording configuration for layer 2/3 (**A1**), layer 5 IT (**A2**) and layer 5 PT (**A3**). (**B**) Average population activity at cue onset early (grey) or late (black) in learning. Shading indicates SEM over neurons (n = 1154, 308 and 560 cells, in layer 2/3 (**B1**), layer 5 IT (**B2**) and layer 5 PT (**B3**)). Green line indicates average of random onsets. Time zero (dashed black line) marks cue onset. (**C**) Average population response to cue onset early or late in learning. Error bar indicates SEM over neurons (n = 1154, 308 and 560 neurons, in layer 2/3 (**C1**), layer 5 IT (**C2**) and layer 5 PT (**C3**)). *, **, ***, p < 0.05, 0.005, 0.0005.

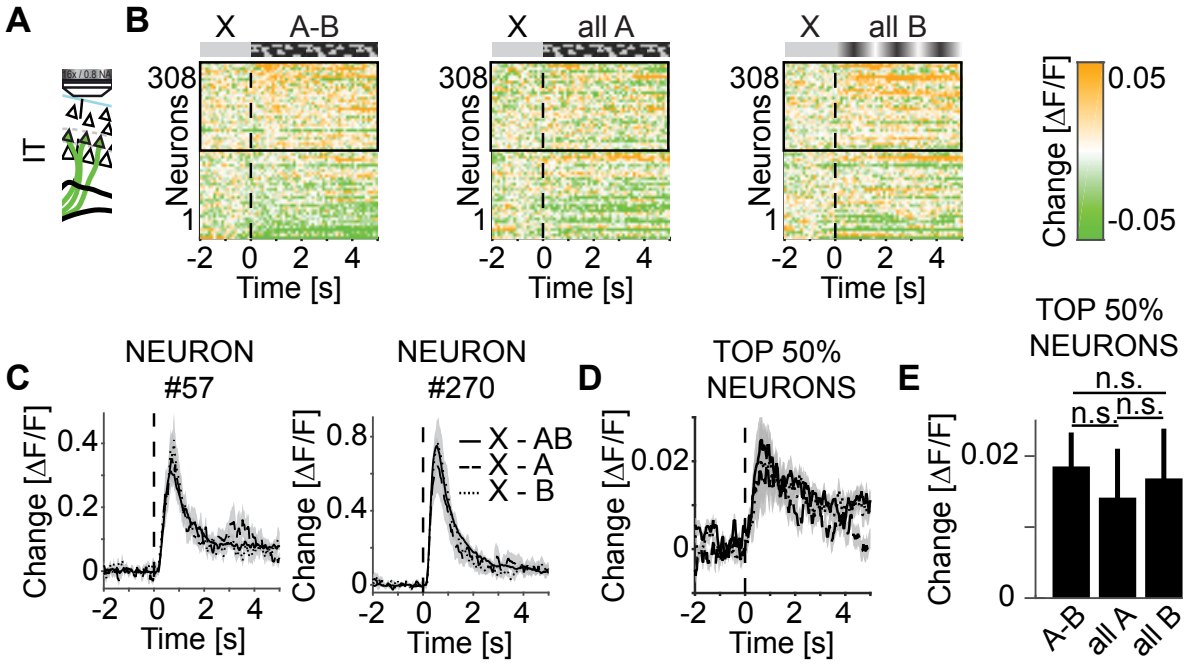


Figure 28. A subset of layer 5 IT neurons is transiently active at visual cue onset

(**A**) Schematic recording configuration in layer 5 IT neurons. (**B**) Average activity of individual layer 5 IT neurons during traversals with different initial visual cues. Time zero marks visual cue onset throughout figure. Box indicates top 50% of neurons. (**C**) Average example neuronal activity during visual cue onset of different types. Shading indicates SEM over trials. (**D**) Average activity of top 50% of neurons during visual cue onset of different types. Shading indicates SEM over neurons (n = 154). (**E**) Average response of top 50% of neurons to different visual cue onset conditions. Note statistical comparisons were pairwise not significantly different, indicating similar activation regardless of cue identity. Error bars indicates SEM over neurons (n = 154).

such instance at which the virtual environment changes informative of task and trial structure was the end of the timeout period, when the virtual tunnel for the current traversal was flashed on-screen.

Indeed, signals could be detected at the end of the timeout period (**Figure 27**). Activity in response to the onset of the virtual tunnel was initially present in all cell types. While in layer 5 IT neurons there was a non-significant trend to an increase in activity over the course of learning, activity declined in layer 2/3 and layer 5 PT neurons (**Figure 27B, C**, early vs zero mean, $p < 0.0005$, 0.05, 0.0005, late vs zero mean, $p > 0.9$, $p < 0.05$, $p > 0.8$, Student's t-test for zero mean, early vs late, $p < 0.005$, $p > 0.3$, $p < 0.005$, paired Student's t-test for layer 2/3, layer 5 IT and PT in $n = 1154$, 308 and 560 neurons, respectively). Thus, only in layer 5 IT neurons did activation at the beginning of the traversal persist throughout learning.

I then elaborated on the IT neuron phenotype by analyzing traversals in which only a single texture was shown as compared to the standard two-texture paradigm. If layer 5 IT neurons responded selectively to the cue onset (rather than the visual identity A or B of the cue), activation in all three trial types, A-B, all A and all B should be similar. Indeed, when I matched the number of events to the different probabilities of their occurrence (**Figure 28B**), individual neurons responded identically, regardless of which texture was actually displayed (**Figure 28C**). This effect prevailed on a population level (**Figure 28D**). I quantified the responses of the top 50% of the most active neurons and found no significant difference in pairwise comparisons (**Figure 28E**, A-B vs all A, $p > 0.1$, A-B vs all B, $p > 0.5$, all A vs all B, $p > 0.1$, $n = 154$ neurons), showing that layer 5 IT neurons are equally active regardless of which texture is displayed.

5.3.8.3 Preparatory activity

In tasks in which animals had to wait for go cues to signal the end of a delay period, activity which rose prior to movement onset had been reported in primates (Churchland et al., 2006; Kaufman et al., 2014) and hypothesized to bring activity to a state which allows for efficient motor execution. This activity prior to movement onset was collectively termed preparatory activity and had also been observed in mice (Li et al., 2015a).

My task did not contain an explicit, reinforced delay period. Instead, mice were free to initiate running at will. I capitalized on those running onset and assessed whether or not preparatory was present and changed in a learning- or cue-dependent manner (**Figure 29**, same data as Figure 19). I quantified activity prior to speed-matched running onsets (t = - 1.125 s – - 0.125 s) and found early in learning a massive increase in activity in layer 2/3 before mice started running. This effect was weaker in layer 5 (**Figure 29E,** VR early vs zero mean, $p < 10^{-59}$, 0.005, 0.05, Student's t-test for zero mean for layer 2/3, layer 5 IT and PT in $n = 1154$, 308 and 560 neurons). Notably, preparatory activity reduced with experience but remained present in layer 2/3 (**Figure**

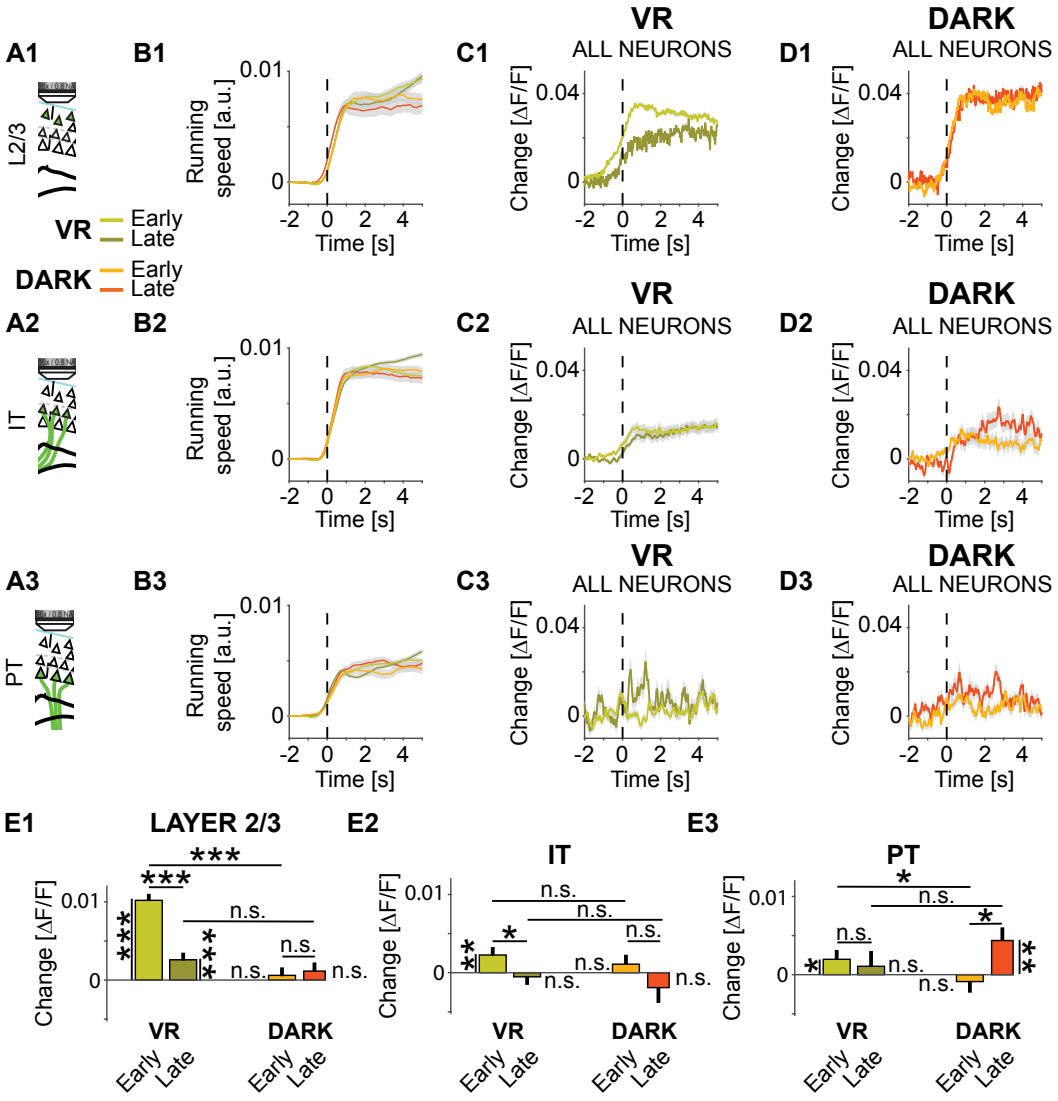


Figure 29. Experience-dependent preparatory activity in layer 2/3 neurons

(**A**) Schematic recording configuration for layer 2/3 (**A1**), layer 5 IT (**A2**) and layer 5 PT (**A3**). (**B**) Average running onset speed matched (see Experimental Procedures) across different conditions and learning stages, VR early (pale yellow), VR late (dark yellow), DARK early (orange) and DARK late (red). Shading indicates SEM over trials. (**C**) Average activity of all neurons during running onsets in VR of the traces selected in (**B**). (**D**) Same as (**C**) but for DARK condition. (**E**) Average population preparatory activity prior to running onset during running in VR or darkness and early or late in learning. Note high preparatory activity in layer 2/3 neurons early in learning which decreased with experience (**E1**).

29E, VR late vs zero mean, $p < 0.0005$, $p > 0.5$, 0.5, Student's t-test for zero mean, VR early vs VR late, $p < 10^{-17}$, 0.05, $p > 0.6$, paired Student's t-test for layer 2/3, layer 5 IT and PT in n = 1154, 308, 560 neurons). I then examined whether the presence or absence of cues (VR vs darkness) influences preparatory activity. I found that only layer 5 PT neurons showed preparatory activity

late during learning though the signal was very noisy (**Figure 29E,** DARK early vs zero mean, $p > 0.4, 0.2, 0.4$, DARK late vs zero mean, $p > 0.2, 0.2, p < 0.005$, Student's t-test for zero mean, DARK early vs late, $p > 0.5, 0.1, p < 0.05$, paired Student's t-test, for layer 2/3, layer 5 IT and PT , n = 1154, 308, 560 neurons). As a consequence, a major difference between VR and darkness conditions could only be found at the early stages of learning in layer 2/3 (**Figure 29E,** VR early vs DARK early, $p < 10^{-23}$, $p > 0.3, p < 0.05$, and VR late vs DARK late, $p > 0.5, 0.4, 0.1$, paired Student's t-test for layer 2/3, layer 5 IT and PT in n = 1154, 308 and 560 neurons).

In summary, preparatory activity was essentially restricted to layer 2/3 neurons. Interestingly, preparatory activity substantially decreased in the course of learning and was absent in darkness, suggesting a role for both experience and cues in motor preparation.

6. DISCUSSION

In this study, I have shown that motor cortex is a critical brain area for mice navigating in a virtual environment and to cope with unpredictable visual feedback. By two-photon imaging, I found activity, throughout the layers, linearly scaled with spontaneously executed turning behavior. This relationship was maintained in layer 5 PT neurons during visual offset perturbation induced corrective turning events but was independent of the subsequent motor behavior in superficial layer 2/3 neurons. During locomotion, activity in layer 2/3 neurons did not scale with the magnitude of behavior. Moreover, I found a multitude of signals to cues relevant for visually-guided navigation behavior in IT neurons as well as reward-related activity dynamics both in layer 2/3 and layer 5 PT neurons.

I will discuss these findings in light of known connectivity and cortical function and, in the concluding section, provide a more conceptualized account in a predictive coding framework.

6.1 Motor cortex is required for virtual navigation

I designed a task in which mice had to navigate in a virtual reality environment in order to obtain rewards (**Figure 4**). Unpredictable visual offset perturbations were introduced to compare spontaneous turning behavior with corrective goal-directed, forced visually guided turning in response to stimuli (**Figure 5**).

Transient inactivation of motor cortical areas can lead to impairments in running, lever-pressing or licking behavior (Komiyama et al., 2010; Otchy et al., 2015; Schneider et al., 2014). Using optogenetic silencing in vGAT:ChR2-EYFP mice I tested if motor cortex was important for virtual navigation. I add to the previous findings by showing that motor cortex is necessary for learning a navigation task (**Figure 6**). Mice did not need to learn novel movements per se; running and turning are natural behaviors which do not require unusual joint kinematics as could be assumed for more complex reaching tasks. It seems more likely that what mice learn is the association between their own movement on the spherical treadmill and the generation of concurrent visual feedback in the virtual environment. The difficulty in learning a new motor skill might lie in dealing with the first few encounters of a problem to which the solution is unknown. By lesioning motor cortex one might deprive animals of their capability to quickly find ways to solve the problem as existing motor primitives have to be meaningfully assembled in a new way to produce a quick response, a capacity subcortical structures might not possess (Kawai et al., 2015; Lopes et al., 2016; Thoroughman and Shadmehr, 2000). From the perspective of evolution, finding a solution quickly might already justify the expansion of cortex. In my experiments, inhibition of motor cortex impaired goal-directed navigation but mice were still able to move (**Figure 6D**) which shows that motor cortex does not command movement in the classical sense.

Brief photoinhibition of motor cortical areas also impaired corrective turning behavior in response to visual offset perturbations (**Figure 7**). This is notably different from a study in rats in which lever-pressing motor behavior, once learned, is unaffected by large-scale physical lesioning of motor cortex (Kawai et al., 2015), but in good agreement with studies that briefly and transiently disrupted motor cortex during similar reaching behavior using optogenetics which found that this method of interference abolished the ability of animals to perform the task (Guo et al., 2015; Otchy et al., 2015). Permanent physical lesions likely engage homeostatic mechanisms which have unpredictable effects throughout the brain which might explain the vastly different phenotypes observed by physical lesions as opposed by acute activity perturbations (Otchy et al., 2015).

I further tested the hypothesis if timing of motor cortical activity was important for corrective turning in response to visual offset perturbations (**Figure 7**) and found that photoinhibition prior to visual offset perturbation onset impaired mice's ability to induce corrective turns to an even greater extent than inhibition concurrent with visual offset perturbation does. My results demonstrate that disrupting motor cortical activity leads to deficits in motor program execution. Moreover, disrupting activity also affects the efficiency of execution of anticipated movements. This suggests that ongoing motor cortical activity shapes the upcoming motor behavior. The hypothesis that motor cortex solely becomes active during movement execution cannot reconcile these results. In instructed-delay tasks a type of neuronal activity has been observed which was coined preparatory activity (Churchland et al., 2006). Disruption of preparatory activity led to longer reaction times (Churchland and Shenoy, 2007). It could be that optogenetic inhibition erased preparatory activity thereby delaying initiation of corrective turning movements. Since visual offset perturbations were random and unpredictable, mice could not have prepared a specific motor program in advance.

The data obtained by shifting photoinhibition stimuli around the onset of the visual perturbation had a bimodal distribution: Either mice initiated a corrective turn or they did not. This provides evidence against the idea that motor cortex activity gradually scales with muscle activity, as was outlined in an introductory section: If I assume that photoinhibition reduces the activity in motor cortex by a significant amount and turning speed linearly depended on activity in motor cortex, then I would have expected to observe a gradual decrease in turning speed. Instead, mice failed to initiate corrective turning altogether (**Figure 7**). Second, motor cortex cannot be solely required for initiation of changes in movement either: Data from the + 1s photoinhibition window demonstrates that, while mice are still able to initiate a corrective turn, the motor program breaks down mid-way for the duration of the photoinhibition stimulus but is resumed after presumably normal activity has been restored. This provides evidence for the idea that normal motor cortical activity is also required during ongoing execution of motor programs.

Another explanation for the observed phenotypes could be found within the framework

sof predictive coding (Rao and Ballard, 1999) and active inference (Adams et al., 2013). Both theories suggest that the brain derives internal models based on the subject's experience in the world (see predictive coding section for a more elaborate account). In brief, active behavior serves to minimize the difference in the predictions of how the world should behave and how it actually behaves (Friston, 2010). This idea implies that the brain continuously compares the predicted and the actual state of the world, even in the absence of changes in movement. If this was the case, then disrupting the activity which underlies this computation should impair movements even when motor programs haven't been initiated yet. This is exactly what I observed by varying the onset window of photostimulation. Mice can probably still perceive the stimulus as their sensory cortices are unaffected. Thus, mice understood some action was required. However, since the comparator computation was disrupted, one strategy that is at least a bit superior to doing nothing would be executing spontaneous behavior which was a sensible solution most of the time mice had to navigate the virtual tunnel.

I fully acknowledge the limitations of the approach. The existence of long-range projecting inhibitory interneurons is known for entorhinal cortex and hippocampus (Melzer et al., 2012) and has recently been demonstrated for motor cortex as well (Rock et al., 2016). Activation of these neurons and thereby inhibiting their projection target area alone might cause the described effects (Otchy et al., 2015). In favor of my experiment it has to be noted that these neurons do not seem to constitute a major output pathway. It thus seems a bigger concern what impact a temporarily shifted balance in feedback projection activity might have (Otchy et al., 2015) than alctual modulation in the long projecting inhibitory neuron's target area. Previous work has shown that optogenetic manipulations onto the surface of the brain because of eminent light scattering, are largely restricted to cortical tissue (Guo et al., 2014). Thus, at least the origin of the deficit due to optogenetic manipulations has to be located in motor cortex.

In summary, the optogenetics data clearly demonstrate a role for motor cortex in virtual navigation both during learning and during induction of corrective turning movements. My data also provide support for the notion that motor cortex actively engages in internal model driven motor control.

6.2 Differences in locomotion- and turning related activity patterns suggest multiple pathways for motor control

I performed two-photon calcium imaging experiments during awake head-fixed mouse behavior and analyzed neuronal activity aligned to different behavioral epochs. I found that activity during spontaneous turns scaled linearly with the speed of the turn. This relationship was independent of learning or lighting condition (**Figure 11 - 13**). Unexpectedly, this was not the case during

locomotion (**Figure 14 – 16**). These different activity patterns might hint at two processing streams for motor control.

A lot of motor cortex research has been devoted to exploring the relationship between neuronal activity and muscle activation or movement direction which might have hampered a more principal understanding of motor cortex function (Scott, 2008; Shenoy et al., 2013). For example, in primate motor cortex the discharge of neurons was found to be well correlated to a preferred movement direction (Georgopoulos et al., 1982; Schwartz et al., 1988), represent various kinematic parameters such as speed (Moran and Schwartz, 1999), joint angular velocities (Reina et al., 2001) or position of the hand during reaching (Wang and Chan, 2007). Studies in cats found good correlations of adjustments in posture or gait and activity of motor cortical neurons (Beloozerova et al., 2003a, 2003b) and imaging in mice found a good correlation of locomotion and neuronal activity (Dombeck et al., 2009). Anatomical data further suggested that motor cortex directly controls motor neurons and hence, muscle activity, possibly by virtue of its direct projection to the spinal cord (Kakei et al., 1999; Rathelot and Strick, 2006). However, using this rationale, it is unclear why turning behavior should engage neuronal ensembles differently (**Figure 21 and 22**) or why the relationship between activity and behavior should differ for locomotion and turning since both movement types activate largely overlapping if not similar muscle patterns.

The picture that emerges is that while the parametric descriptions are valid, motor cortex cannot be the sole driver of movement (see section on optogenetics) but has to fulfill other functions. One possibility is that motor cortex is the main source of efference copies used to generate predictions of sensory percepts of our environment (see predictive coding section below for a fuller account).

Based on the data I obtained, in a more conventional framework, I speculate that two processing streams be present in motor cortex which likely multiplex (many locomotion-related cells are also active during turning behavior). These two streams could be mediating motor programs for “hold” and “move” activity, as recently suggested (Shadmehr, 2017). In brief, Shadmehr postulates that during goal-directed movements, there could be a circuit which mediates the actual movement period and, when required to maintain the body state after movement, there is a separate circuit mediating the sustained activation of the required muscles. A study in primates indeed showed that motor cortical cells are transiently active during initiation of a reach movement and, when monkeys were required to keep their hand at the target position, spinal interneuron showed persistent activity (Shalit et al., 2012). In my data, locomotion onset related activity persisted throughout the locomotor bouts in layer 2/3 and layer 5 IT neurons but was phasic in layer 5 PT neurons (**Figure 14, 19**). Notably, turning-related activity was phasic in all cell types. Activity of layer 5 PT neurons therefore was in good agreement with the previously mentioned study (Shalit et al., 2012). But why would locomotion-related activity be persistent in layer 2/3 and layer 5 IT neurons? One potential explanation could be that activation patterns in

upper motor cortex layers are unique depending on the movement type (rather than mapping onto individual cells). Together with the observation that turning-related activity was phasic and therefore “move”-like, it suggests a separate pathway for either locomotion or turning behavior. A third option could be that activity appeared to be persistent due to slow calcium indicator dynamics or many cells tiled a locomotor bout by transient activity which on the population level appeared elevated throughout the movement period. This, however, was unlikely: Brief phasic activity on the timescale of what I observed in PT neurons would have easily been detectable in upper layer neurons, too. Moreover, individual cells appeared to have prolonged activity which is incompatible with the tiling hypothesis. Note that this discussion largely ignores the distinction between inhibitory and excitatory neurons which would be expected to fall into the opposite categories, that is: a “hold”-like persistently active inhibitory interneuron would actually serve to terminate “hold”-like activity and thereby aid the function of “move”-type activity.

From a behavioral perspective turning-related activity should be “move”-like, that is: phasic. If mice were turning in a perfectly goal-directed manner, the optimal turn will never exceed a half circle in one direction. Thus, while there are situations in which forward locomotion needs to be sustained, perhaps without foreseeing for how long, turning in any case is a movement type with a well-defined endpoint which only needs to be performed transiently. Hence, the most sensible activation pattern is the observed phasic, “move”-like one.

6.3 Activity in layer 2/3 neurons resembles motor plans and activity in layer 5 PT neurons could drive motor behavior

A major goal of this study was to compare activity during spontaneously initiated motor behavior with activity during induced, visually-guided behavior in response to unexpected events. I found that in reaction to unpredictable changes of visual feedback during visual offset perturbations, neuronal activity in layer 2/3 and layer 5 IT appeared stimulus-driven while activity in layer 5 PT neurons retained a linear relationship with turning behavior, that is: I observed more activity the higher the turning speed of the mice was (**Figure 20**). I speculate that activity in layer 2/3 might reflect motor plans whereas activity in layer 5 PT neurons drives the upcoming motor behavior.

Activity which scaled with behavior had been described in corticofugal motor cortex neurons (Beloozerova et al., 2003b) and, more recently activity in rat frontal orienting field (FOF), an area in which the activity has been suggested to give rise to motor plans, was found to correlate better with the upcoming motor act than the sensory cue that was presented in order to trigger the movement (Erlich et al., 2011). Using electrophysiology and considering the fact that layer

5 neurons were reportedly more active than superficial layer neurons (O'Connor et al., 2010), it is conceivable that the study in rat FOF mainly recorded from layer 5 PT – like neurons. This would provide support to the notion that activity in layer 5 PT neurons reflected upcoming motor actions. If this was true, these neurons would need to show specificity with regard to what the mice are doing, that is: they should be specific to the turning direction. Supporting this view, layer 5 neurons in rat motor cortex were found to be very selective for ipsi- or contraversive turning movements (Gage et al., 2010). Consistent with the literature, in my data, activity of layer 5 PT neurons can also be considered reflective of the motor behavior: For example, layer 5 PT neurons which are active during a preferred spontaneous turn direction were specifically active during induced turns only when mice were turning in the initially preferred direction of the cell (**Figure 22**).

Sensory signals, along with a plethora of other responses (e.g. preparatory activity (Churchland et al., 2006)), have been reported in primate premotor and motor cortex (Alexander and Crutcher, 1990; Cisek and Kalaska, 2005; Hatsopoulos and Suminski, 2011; Merchant et al., 2001; Rao and Donoghue, 2014; Salinas and Romo, 1998; Shen and Alexander, 1997b; Shen and Alexander, 1997a). In mice, using Channelrhodopsin-assisted circuit mapping, it was subsequently found that sensory related activity is preferentially routed to upper rather than lower motor cortical layers providing a means of how stimulus-driven layer 2/3 activity could come about (Hooks et al., 2013; Huber et al., 2012; Mao et al., 2011; Murray and Keller, 2011; Suter and Shepherd, 2015). However, these studies avoid the question what, in local motor cortex, the purpose of sensory-driven activity might be. Moreover, given that the majority of layer 5 PT neuron input stems from layer 2/3 and layer 5 IT neurons (Anderson et al., 2010; Weiler et al., 2008) and together with the fact that the reciprocal connectivity motif of layer 5 PT to upper layers is not existent (Kiritani et al., 2012), this suggests that the motor behavior correlated activity in layer 5 PT neurons is largely derived from the activity pattern of layer 2/3 and layer 5 IT neurons. This makes the question what the significance of stimulus-driven activity in upper layers is even more pressing.

I found activity in layer 2/3 neurons which signaled the presence of a stimulus rather than direction of a movement required to react to the stimulus. Accordingly, neuronal activity in layer 2/3 was independent of the vigor of movement (**Figure 20**) and was therefore not reminiscent of a motor command in the classical sense (meaning capable of driving muscle activity). The hypothesis that motor cortex would generate movement commands in the classical sense I disagreed with earlier already. Finally, decoding layer 2/3 neuronal activity, because of the aforementioned features, would not be able to predict the behavioral response of the mice.

Activation in layer 2/3 neurons, if sensory, had to be derived from the visual modality as this was the only input that was manipulated during visual perturbations. A study mapped responses in motor cortex to pure visual stimuli while primates were fixating their gaze (Merchant et al., 2001). They found a number of neurons which were active in the absence of movement.

However, if the visually driven activity was a result of direct input from the visual system, one might assume that neurons in motor cortex inherit receptive field properties from visual system neurons. Interestingly but not unexpectedly, this was not the case (neurons responded regardless of the direction of the visual offset, **Figure 22**), suggesting that, while "visually driven" is a valid description of the responses, the actual function these neurons subserve in motor cortex might be otherwise (see predictive coding section for an alternative view).

Erlich et al. speculate that rat FOF may provide motor cortex with motor plans which are then executed (Erlich et al., 2011). My data rather suggests that activity in superficial layer 2/3 and layer 5 IT neurons of motor cortex itself might play the role of deriving motor plans. Supporting this idea, a recent paper in mouse ALM cortex, using a paradigm of instructed delay forced-choice licking, proposed layer 5 IT neurons to play a key role in motor planning (Li et al., 2015a). Moreover, in a study carried out in primates a color indicated the type of the upcoming task. The authors found that neurons in motor cortex became responsive to the cues but their activity was otherwise uninformative of movement direction (Zach et al., 2008). The authors further claim that the response properties were acquired in the course of learning, on a rapid timescale, when the cues were behaviorally relevant. This is roughly similar to what I observed during visual offset perturbations in layer 2/3 because activity in these neurons was also uninformative with respect to the turning behavior but extends the current knowledge and could be interpreted in terms of learned motor plans. Unfortunately, I introduced visual offset perturbations only from day 3 on which precludes any statement if stimulus-driven activity was present already before learning. However, based on the study by Zach et al., I would assume these activity patterns are learned and therefore not present at the beginning of training (also see predictive coding section for another argument why activity during visual offset perturbations should be learning-dependent).

My data suggests that during visual offset perturbations, sensory driven superficial activity is transformed into activity in deep layers which could drive motor effectors. This computation has been termed sensorimotor transformation and has been observed in primate motor cortical areas (Rao and Donoghue, 2014; Shen and Alexander, 1997; Zhang et al., 1997). As mentioned previously, connectivity between superficial layers and layer 5 PT neurons is largely unidirectional (Kiritani et al., 2012) and the majority of deep layer input is derived from intracolumnar sources (Anderson et al., 2010; Weiler et al., 2008). My findings therefore add to the current knowledge because it would strongly suggest that in mouse motor cortex, the intralaminar projection from superficial layers to deep layer 5 PT neurons is the site of sensorimotor transformation (but see predictive coding section for an extended conceptual idea on this). Finally, the literature frequently acknowledges brain structures supplying motor plans to primary motor cortex (e.g. Erlich et al., 2011; Rao and Donoghue, 2014). It could be that, in the course of evolution, while organisms had to cope with more complex environments, the generation of motor programs was "outsourced" to multiple brain areas. Nevertheless, my data indicates that in mice, layer 2/3 neurons of local motor cortex might fulfill this function by themselves already.

Why could layer 2/3 neuronal activity resemble motor plans? When faced with an unexpected visual offset, mice have to reassess the situation and quickly derive a behavioral response. In order to avoid passivity, a first sensible approximation of an appropriate behavioral response might be relying on behaviors that have previously been experienced to be useful: spontaneous turns which helped navigating the virtual tunnel. It could be that motor cortex, in order to derive a behavioral response, first activates motor programs for either spontaneous turn. In this case, neurons would initially be expected to be co-active to about equal degree, a pattern which I observed (**Figure 22 and 23**). The co-activation of both cell groups might help save computation time to be able to quickly react. Reaction times that I observed were in the range of ~ 200 ms, which is very fast considering transduction delays and the fact that a motor program has first to be derived. On the other hand, I rarely observed wrong behavior, indicating that the correct behavioral response is routinely chosen based of the ambiguous motor plans derived by layer 2/3 circuits. In layer 5 PT neurons this ambiguity could be resolved by selecting the correct motor plan which is then subsequently executed.

6.4 Learning-related changes suggest strengthening of motor cortex output layers for more efficient execution of behavior

Capitalizing on the chronic recordings, I analyzed responses of motor cortical neurons lamina-specificly either early or late during learning for two movement types, spontaneous turns (**Figures 17 and 18**) and self-initiated locomotion (**Figure 19**). While the relationship between activity and behavior was learning independent for either movement type (**Figures 12 and 15**), there was a learning-related increase in the vigor by which mice initiated movements (**Figure 5**) which also depended on the condition (VR or DARK) the mice were behaving in. Thus, I further detailed the analysis by speed-matching individual conditions (**Figures 18 and 19**, early and late and VR and DARK).

For turning related activity (**Figure 17**), I found that while activation decreased in the course of learning in layer 2/3 neurons for both ipsi- and contraversive turns, it increased in layer 5 PT neurons during contraversive turns. Furthermore, there was no bias for either turning direction in layer 2/3 neurons either early or late in training. In layer 5 PT neurons, however, the bias for activity during contraversive turns became larger. Activity in layer 5 IT neurons was neither biased nor changed in a learning-dependent manner. This pattern held largely true when comparing turns in VR and DARK conditions with the notable exception that in layer 5 PT neurons, turning related activity in DARK was significantly lower than under VR conditions (**Figure 18**).

This pattern was also similar when considering self-initiated locomotion onsets (**Figure 19**) as during speed-matched running onsets in VR. Activation became less in layer 2/3 neurons, remained unchanged in layer 5 IT neurons but became larger in layer 5 PT neurons. Interestingly, in layer 2/3 neurons, when analyzing running onsets in the DARK condition, activation was not learning-dependent. These data clearly show that activity in motor cortex circuits is plastic during multiple behaviors.

It had previously been shown that activity correlates with and changes in a directional manner for behavioral task related variables (Huber et al., 2012; Komiyama et al., 2010) and the prediction accuracy of behavior from recorded activity becomes better in deep layer neurons (Masamizu et al., 2014). Activity in deep layer PT neurons was shown to be biased to contralateral movements and presumably drives behavior (Li et al., 2015a) but how this bias arises was unknown. I can close this gap of understanding partly by showing that the activity bias in layer 5 PT neurons during contraversive spontaneous turns, although already present in the early stages of learning, was increased even further due to a selective increase in activation (**Figure 17**). My data does not support the possibility that activity during ipsiversive turns becomes sparser. For this study, all activity recordings were carried out in the right hemisphere, thus the description contraversive turn refers to left turns. It seems likely that when recording in the left hemisphere, activation in layer 5 PT neurons would be biased towards right turns.

Activation during running onsets in VR decreased with learning in layer 2/3 neurons (**Figure 19**). An earlier study found a good correlation of subsets of neurons in motor cortex with locomotion behavior, but, due to technical limitations at the time, the authors were not able to determine long-term changes in neuronal activation (Dombeck et al., 2009). Subsequently, studies have focused on forelimb or licking motor behavior (Guo et al., 2014; Huber et al., 2012; Komiyama et al., 2010; Li et al., 2015a; Masamizu et al., 2014), presumably under the assumption that more complex tasks provide richer activity patterns from which to derive conclusions, but activity during simple locomotion behavior in mice has been largely ignored since then. I am therefore not aware of studies that would contradict the learning-related decrease in activity during locomotion onset that was observed here.

Given an almost inverse relationship between activity and vigor of locomotion behavior (**Figure 14**) and, together with the fact that in the course of learning movement becomes more vigorous (**Figure 5**), these findings are not unexpected and this was the reason why I further analyzed the activity by speed-matching locomotion onsets across conditions (**Figure 19**). It appears as if a reduction in preparatory activity in layer 2/3 can almost fully account for the learning-related decrease in activity during locomotion onsets (see next section below). This was interesting because it suggests that rather than a change in behavior, an internally-driven change in state is the reason for the observed decrease in activity over the course of learning. Additionally, learning dynamics during locomotion onset were notably different in layer 2/3

neurons when mice where running in darkness as compared to when mice were navigating in VR conditions: Neither was I able to detect a decrease of activation in the course of learning nor was there substantial preparatory activity present during either stage of learning, but overall activity during locomotion onsets tended to be higher in darkness than in VR (**Figure 19**). Since locomotion behavior was speed-matched for all conditions, these differences cannot be explained merely by a systematic, learning-related change in behavior. Instead, internal dynamics must be the underlying reason. If this was true, then, quite remarkably, the use of an internal model as evidenced by preparatory activity in the early stages of learning, is dependent on the lighting condition. Along a similar line of reasoning, removal or obstruction of visual feedback has often been used in human psychophysics to probe internal models for reaching behavior (e.g. Gallivan et al., 2016, 2015; Stewart et al., 2014); in the concluding section I will suggest a predictive coding scheme to reconcile this data.

Changes in neuronal firing rates have also been observed in primates during learning of motor tasks (Gandolfo et al., 2000; Li et al., 2001; Paz et al., 2003). However, because these recordings have been carried out using electrophysiological methods, these studies have not been able to distinguish the laminar position or projectional identity of the recorded cells. My data therefore substantially adds to the existing knowledge by showing that learning related changes are of opposing dynamics for superficial layer 2/3 and deep layer 5 PT neurons. The pattern of learning-related changes I observed for locomotion and turning related activity (**Figures 18 and 19**) would be consistent with the idea that during consolidation of a motor skill, activation shifts from layer 2/3 to deep layers in order to enable new plasticity in associative superficial layers.

6.5 Multiple non-motor related signals implicate motor cortex in a broad range of functions

The plethora of signals found in motor cortical physiology has always been difficult to reconcile. As cited in previous sections of this discussion already, numerous studies found representation of movement- and non-movement related parameters in motor cortex. This is likely a major reason why a general consensus for a framework of cortical motor control has not been agreed upon to date (Graziano, 2006; Hatsopoulos and Suminski, 2011; Scott, 2008; Shenoy et al., 2013). I, too, find a variety of signals which I will discuss here and in a more conceptualized manner in the following section.

6.5.1 Preparatory activity in layer 2/3 is learning dependent

Preparatory activity, by definition, occurs in the absence of movement. I found that preparatory

activity in layer 2/3 neurons was greatly reduced in the course of learning (**Figure 29**). Thus, the actual movement-related activity in layer 2/3 neurons during running onsets in VR is presumably much lower, perhaps in the range of the late learning stage. Conversely, this would imply that already in early VR stages, the movement-related activity during running onsets is substantially higher – about 2-fold - in darkness than in VR. Moreover, clear preparatory activity was absent in darkness. Considering statistically indistinguishable motor behavior, this warrants explanation. A predictive coding account would assume that initially, at early learning stages, internal models have to be updated and adjusted to the new task (navigation in a virtual reality system). This might require more preparatory activity as compared to navigation in late stages of learning since the adjustment of the internal model has to take into account uncertainties arising from the new situation which requires the circuit to be more active. At later stages, when the internal model crystallized, this additional activity is not needed anymore and is eliminated (**Figure 19C1**). In darkness, however, uncertainties of how to achieve the goal of the task are much higher because visual cues are lacking. This condition also does not change with learning since in darkness the predictability of the environment does not increase even with increasing navigation expertise of the mice. Hence, an equal amount of activity is required for running in darkness at either stage of learning, early or late. In summary, learning-related activity differences in layer 2/3 during running onsets in VR might reflect the adjustment of predictive activity necessary to update internal models.

6.5.2 Local reward-related activity might act as a reinforcement signal for motor learning

Learning theories generally assume that a (teaching) signal be present in order to reinforce advantageous behavior (Bush and Mosteller, 1951; Suri, 2002; Sutton, 1988). The learning related activity patterns discussed earlier naturally raise the question if such a teaching signal can be found in local motor cortex.

I have found reward-related activity most prominently in layer 2/3 and layer 5 PT neurons (**Figure 24**). Activation ramped up seconds prior to reward delivery in the VR condition suggesting reward anticipatory activity. However, it was difficult to disentangle activity of reward anticipation from anticipatory licking related activity. More telling in this respect was the activation in response to reward delivery in darkness. Neurons in layer 2/3 and layer 5 PT swiftly increased activity upon reward delivery; moreover, this activation was increased during learning in layer 5 PT neurons.

Ramping of activity in anticipation of a reward would be exactly what temporal difference learning models would predict for activity of VTA dopamine neurons (Suri and Schultz, 2001). In rat motor cortex, studies have found preferential innervation of dopaminergic VTA fibers in

deep layers and their presence was critical for motor learning (Hosp et al., 2011; Molina-Luna et al., 2009). It seems conceivable that the ramp-up observed in layer 5 PT neurons is a direct effect of dopamine-mediated elevation of activity; the ramp observed in layer 2/3 neurons could be mediated by a polysynaptic pathway. In that respect, it was interesting that activity during reward delivery was dependent on learning stage only in layer 5 PT neurons: It supported the notion that layer 5 PT neurons might be the cell type which directly receives teaching signal-like dopaminergic input.

6.5.3 Activity in layer 5 IT neurons can be driven by cues in the virtual environment

In most analyses in which layer 2/3 and layer 5 PT neuronal activation differed the activation of layer 5 IT neurons was unchanged, with the notable exception of analyses of cue-driven activity. A largely non-overlapping subset of layer 5 IT neurons was strongly and specifically driven by either the texture change occurring at the midpoint of the tunnel (**Figure 25 and 26**) or the onset of the visual cue at the beginning of each traversal, after the timeout (**Figure 27 and 28**).

More broadly speaking, layer 5 IT neurons were driven by cues which allowed mice to predict the reward but did not respond strongly during reward delivery itself (**Figure 24B**). This notion was supported by the fact that the activation was learning dependent for both the texture change and the visual cue onset. Layer 5 IT neurons can broadly sample input from and send output to the entire telencephalon (Harris and Mrsic-Flogel, 2013; Shepherd, 2013). Visual input could therefore be derived from visual areas. Layer 5 IT neurons might also receive input from PPC, an area in which activity has been shown to contain sequence-specific information which could be used to predict behavioral choices for reward collection (Harvey et al., 2012).

Subsets of neurons responding to either cue (visual cue onset or texture change) were largely non-overlapping. That was interesting because both cues were visual in nature. Thus, while the driving signal could be originating in visual areas, it would still require specific connectivity in order to drive neurons in the context of one reward-predicting visual stimulus but not the other. Activity in layer 5 IT neurons has previously been noted to be exquisitely stimulus-specific (Turner and DeLong, 2000). My virtual environment, however, did not cover and was not meant to cover a rich stimulus space. Extrapolating from the high degree of stimulus-specificity – a slightly different visual stimulus (visual cue onset or texture change) drives non-overlapping subsets of cells – it is conceivable that layer 5 IT neurons might become much more active in a selective manner if there were other reward-predicting stimuli present in the environment. One function of layer 5 IT neurons could therefore be representing and processing stimuli which predict upcoming rewards.

6.6 A predictive coding account of motor control

Much research has been devoted to defining receptive fields of neurons, especially in the sensory system since in this domain stimuli are well controllable. However, this becomes a notion that is particularly difficult to reconcile when trying to conceptualize neuronal activity in high-level structures of the motor system such as motor cortex because the brain's function is likely to orchestrate movement rather than representing it.

Predictive coding is a theory of cortical function (Rao and Ballard, 1999) built on the observation that anatomically, circuit motifs are of similar design, which has widely been recognized and, among other names, been termed cortical column or canonical circuits (Harris and Shepherd, 2015). It provides a principled explanation for the hierarchical structure of cortical processing and what information feed-back (or: top-down) projections could be transmitting. In predictive coding schemes, feed-forward (also: bottom-up) connections of any type (e.g.: thalamo-cortical) are assumed to convey prediction errors computed as the difference of the input from the hierarchically lower structure and the feed-back projection from hierarchically higher areas. Feed-back projections are thus thought to convey predictions about the current state of their target structure. Local circuits function as comparators and, depending on the synaptic weights of the inputs, as filters, for lower-level feed-forward and high-level feed-back projections. [This could beg the question how cortical regions relate to each other or where the "turning point" in the hierarchy is, e.g. which structure is not origin of feed-forward projections. However, this is a conceptual problem only if motor cortical projections are referred to as feed-forward (in a sense of: "driving") rather than top-down or feed-back which is the point of view of this section of the discussion.] Sparse coding can be explained in this framework because each level of the cortical hierarchy only becomes active once there is a *difference* between its inputs (not upon input per se), thereby dramatically reducing neuronal activity (and perhaps enabling the existence of such densely packed neuronal structures in the first place). More generally, predictive coding has been invoked to explain perception as an active process which is heavily influenced by prior beliefs (Friston, 2010). Friston suggested that the fundamental goal of the brain might be to minimize prediction errors which could be achieved in two ways:

1) By updating one's own prediction of the sensory stimuli in the world.

2) By taking action such that sensory stimuli align with the internal expectation.

For the first postulate, predictive coding has gained momentum to explain extra-classical receptive fields in sensory cortical areas (Fiser et al., 2016; Keller et al., 2012; Rao and Ballard, 1999; Zmarz and Keller, 2016). A core idea is the internal model, a learned, experience-dependent approximation of the external world which is used to form expectations and predict the sensory consequences of motor actions. The cerebellum, among other structures, is believed to be the brain structure that contains sensorimotor internal models (Caligiore et al., 2016; Miall and

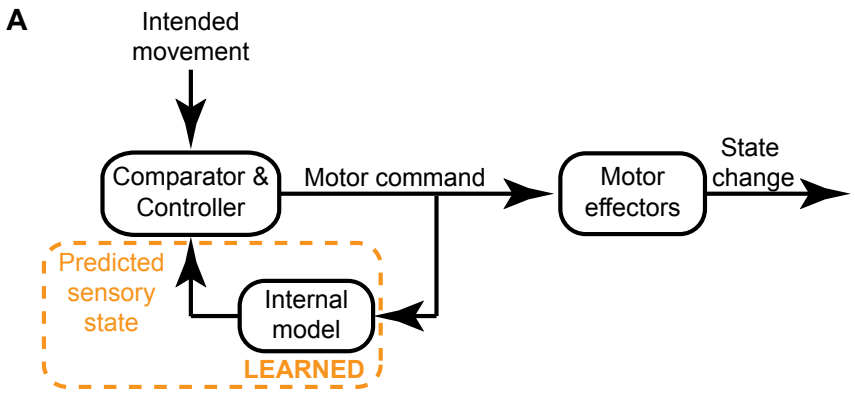
Wolpert, 1996; Tseng et al., 2007).

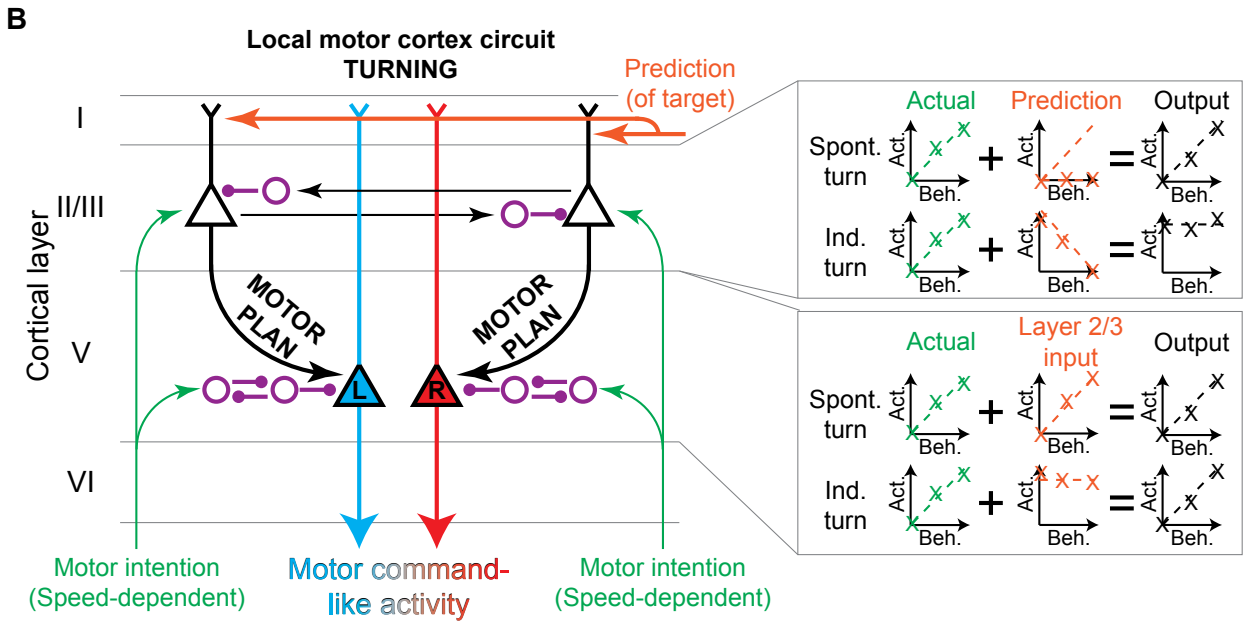
In support of the second postulate, motor control (= the mechanisms by which action is generated), has been recognized to be predictive in a sense that transduction delays both from the sensory periphery which inform about the current state of the body and delays until the motor command-like activity arrives at its effector motor neurons have to be taken into account for executing goal-directed movement (Miall and Wolpert, 1996). Thus, the brain has to ensure that movements are appropriately executed at a time point at least 50 ms (minimal transduction delay) into the future and not at the time when the sensory periphery first detected a change in the environment requiring movement. Predictive mechanisms are good candidates as they do not rely on immediate sensory feedback and can therefore be implemented at much higher apparent speed. Despite these parallels between predictive coding and motor control phenotypes, predictive coding schemes have rarely been used to explain neuronal activity encountered in motor cortical areas.

Numerous human psychophysical studies have shown that motor control during reaching relies on internal models (Franklin and Wolpert, 2008; Gallivan et al., 2016b, 2015; Haruno and Wolpert, 1999; Kluzik et al., 2008; Tseng et al., 2007) and animal species as low in the evolutionary tree as dragon flies (Mischiati et al., 2014) and salamanders (Borghuis and Leonardo, 2015) employ internal models for generating movement. It is reasonable to assume that mice also employ internal models and, given the extensive cortico-thalamo-cerebellar loops described in the introduction, predictive coding schemes could heavily influence motor cortical processing. Questions of where and how, on a cellular level, predictive mechanisms are implemented have, however, remained largely unaddressed.

In predictive coding, a fundamental computation is the comparison of feed-back prediction and feed-forward error. Thus, if neuronal activity was reflective of a predictive coding framework, it could either take the shape of expectations (predictions) – that is: the believe about the state of the system given current input – or deviations (= errors) of the current state from the expected state. The outcome of this computation should update both the actual motor program and the internal model of the movement's next execution via efference copy. Components of this circuit have been described in auditory (Keller and Hahnloser, 2009) and visual (Keller et al., 2012) sensory systems. Owing to the lack of a clear stimulus, in motor control the distinction between what would be expected and what is experienced is less sharp. I would like to put forward 3 claims derived from the previous thinking which I will try to provide support for subsequently:

Claim 1: Motor cortex could compare the ongoing (expected) motor program with what would actually be necessary (desired) to achieve a goal in the face of an unpredicted perturbing event. The activity could then be reflective of the difference of the two – expected and desired - motor programs, that is: the prediction error. Hints at such a computation have been obtained in primates (Inoue et al., 2016).





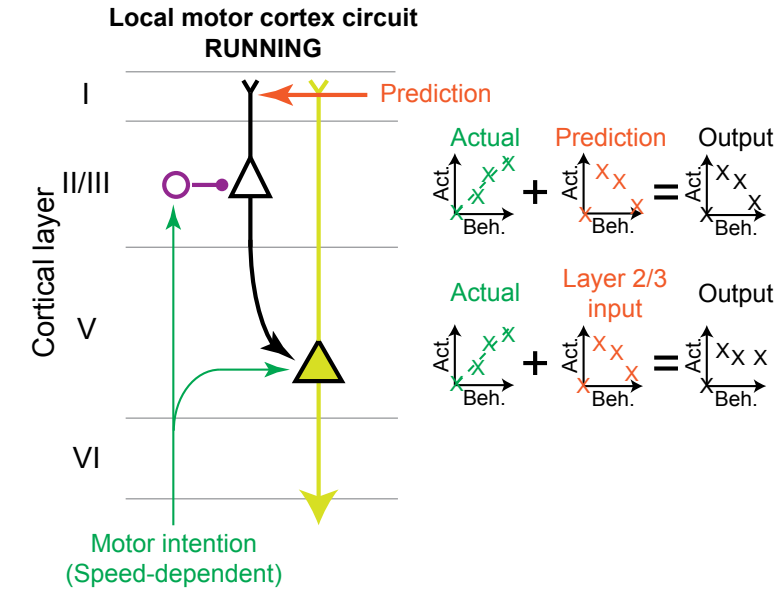


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Figure 30. Models and circuits for predictive motor control

(**A**) Classic robotics successively produces incremental motor commands which lead to state changes. State changes are assessed by sensors and their output signal can be utilized to generate new motor commands. Despite much effort, modern robots routinely fail to react adaptively to unexpected novel situations. Animals can use efference copies of motor commands to update internal models which are used to generate a predicted sensory state. This prediction is speculated to be compared with the movement intention to generate appropriate output. The generation of predictions derived off the internal model is an experience-dependent process which likely influences the entirety of motor control.

(**B**) I speculate that comparator and controller entities in (**A**) map onto layer 2/3 and layer 5 PT neurons during turning, respectively. Layer 2/3 might compare internal model-generated predictions, possibly stored in the cerebellum, with the intention of movement, thereby generating motor plans. Layer 5 PT neurons select and execute motor programs. Their activity again mimics predictions which are sent to the spinal cord for translation into motor effector-competent activity (Adams et al., 2013). Alternatively, the connectivity between layer 2/3 and layer 5 serves as the substrate to perform a sensorimotor transformation allowing layer 5 PT neurons to directly send motor effector-competent activity. Local interneurons serve to gate activity during specific movement epochs. Likely, multiple such movement generating circuits are arranged in parallel to process different internal models.

(**C**) Circuit schematics alike to (**B**) but for locomotion.

Claim 2: Activity depends on the context and is situational. For example, the history of previously encountered events which determines the statistics of the environment is crucial to the ability to predict. It follows that predictions and signals related to them do not only depend on the precise situation (e.g. one is very unlikely to encounter the exact same situation multiple times) but are also learning and experience-dependent. In contrast, if one was to execute identical movements either as a child or as an adult, pure motor commands should look identical (which they do not, see e.g. Raptis et al., 2010). That is, activity reflecting pure motor commands should be context-invariant.

Claim 3: The brain's goal is to minimize prediction errors. This would be advantageous from an evolutionary perspective in that biological implementations which require the least amount of energy are preferred. Because in a predictive coding framework processing stages only need to represent differences of their inputs, the required amount of energy is vastly reduced up to a point of non-activity.

6.6.1 The goal of sensorimotor learning could be to finesse predictions

To a certain degree, the brain's circuits need to be functionally hardwired. Frequently encountered

problems in the natural environment should preferably not be subject to lengthy inference and rather have to be dealt with swiftly and efficiently by using memorized solutions (Rauss and Pourtois, 2013). A prime example for hardwired architectures are brainstem and spinal motor circuits. Lesioning elements therein or interrupting communication between those motor regions and motor effectors results in severer deficits than lesioning an equal volume of cortical tissue, also in untrained animals. Cortical circuits might be needed for error correction during behaviors that cannot be easily dealt with using hardwired circuits such as inferring movement strategies in novel (virtual) environments where internal models of the physics of this world first need to be learned and then continuously updated and refined (Lopes et al., 2016). For mice learning to navigate the navigation task in this study, the virtual environment was clearly novel and at first unpredictable. Mice primarily have to learn the association of their own movement and the feedback in the virtual environment which can also be described as a process of updating the internal model. Disrupting this process likely has a big impact on the ability to efficiently execute movement during unexpected events which is consistent with what I found using targeted photoinhibition (**Figure 6**).

Learning an internal model could be reflected in more efficient execution of the respective behavior, an effect that is routinely observed during motor learning (Cohen and Nicolelis, 2004; Kimura et al., 2012; Komiyama et al., 2010; Masamizu et al., 2014; Petreanu et al., 2012) and has also been observed in this study (**Figure 5**). Visual offset perturbation onsets were also random implying that the exact same stimulus situations (e.g. viewing angle or running speed might differ, auditory background noise might vary) could not have happened twice. Nevertheless, corrective motor behavior was precise and adaptive, suggesting that rather than simply re-using a previously learned motor sequence, mice built on a generalized representation of the virtual environment to efficiently perform the task. Moreover, during timed photoinhibition experiments, I found that the ability of mice to successfully execute corrective movements was impaired to a greater extent when the inhibitory stimulus preceded the visual offset perturbation stimulus as compared to concurrent photoinhibition (**Figure 7**). It could be that prior photoinhibition eliminates the currently applied internal model for virtual navigating and its erasure prevents mice from inducing the corrective turn. In summary, the results presented here could support the notion that the actual goal of motor learning is forming and finessing predictions (and not to expertly execute a given sequence) which are then used to minimize errors through active behavior.

Different susceptibility to motor cortical lesions across species could be due to their differential reliance on cues. Rodents, for example, use primarily proximal cues such as somatosensory ones for coping with the environment. However, when rodents were challenged in an unfamiliar virtual environments, acute photoinhibition of motor cortical areas still has a profound impact, consistent with the idea that in settings where internal models need to be updated, motor cortex is needed (Guo et al., 2015). In contrast, primates, which had to navigate tree tops and therefore an elaborate 3-dimensional environment, cannot exclusively rely on the

sense of touch but rather have to capitalize on senses allowing to evaluate distal cues, such as vision. Thus, if motor cortex was to integrate contextual cues in an internal model to generate movement, motor cortical lesions would have vastly bigger impacts in species employing complex internal models explaining the apparent discrepancy in the literature (see Introduction section).

6.6.2 Activity in motor cortex complies with predictive coding schemes

6.6.2.1 Activity during turning

Predictive coding posits that comparator circuits be present which compute the difference between predicted and actual input (of any type). Displacement of the target results in a sensory error present as the difference between where the brain expects the target to be and where it actually was located. This visual error is hypothesized to drive motor adaptation (Miall and Wolpert, 1996) and corrective turns could be the behavioral correlates in this task. The optogenetics experiments (**Figure 6 – 10**) have shown that motor cortex is involved in learning of the task and corrective turning. With the activity recordings, I attempted to understand the computations underlying the behavior. Supporting the idea that motor cortex might be site of internal model learning, using disruption of motor cortex activity by repetitive transcranial magnetic stimulation in humans, it has been suggested that internal models are retained in M1 after motor learning (Cothros et al., 2006).

Mice, as they navigate the virtual tunnel, can capitalize on existent movements: Motor commands (**Figure 30A**) do not have to be learned anew, hence the known relationship between motor command activity and motor effectors producing state changes can be re-used. Classic robotics would assume that, after a state change has happened, external sensors collect information about the new state and relay this information back to control centers. This is clearly not the way movements are controlled since the collection of information before the next movement step would cause long transduction delays which is not the way animals behave. Instead, likely an efference copy of the motor command is used to update internal models in which predictions are stored of what the new state should be given the employed motor command. The output of the internal model - a predicted sensory state - is then used to compare with the intended movement and generate input to the controller. I would like to speculate in the following that comparator and controller map onto either layer 2/3 and layer 5 PT units, respectively (**Figure 30B**).

During learning, the likely site of plasticity is the internal model of the environment. Mice learn the physics of the environment and the way by which their own movement changes the virtual environment. Thus, in the course of learning, the predictability of the environment increases alongside with the efficiency of motor behavior execution. If layer 2/3 circuits compute

the deviation between intended motor program and the prediction of its consequences (**Figure 30B**) this deviation should become less with learning because the sensory consequences of the mice's movement are as expected. In other words: As predictability increases, the error between prediction and the outcome of actions is reduced, in line with current theories of brain function (Friston, 2010). Thus, if layer 2/3 neurons were such error units, their activity should decrease over the course of learning. This is exactly what I observed. In parallel, on a similar timescale, units which provide predictions would increase their activity as predictions become refined and the predictive power of the internal model increases. Cell types which convey predictions should therefore be more active at late stages of learning. This maps well onto layer 5 PT neurons (**Figure 17)**. In summary, layer 2/3 neurons in motor cortex could functionally mediate error computations in a predictive coding framework while layer 5 PT neurons, those thought to mediate the motor output, are the neurons conveying predictions.

Motor command-like activity driving motor adaptation will be indistinguishable from prediction signals which might update internal models. As previously elaborated, the cerebellum is a likely site for storage and maintenance of internal models. Layer 5 PT neurons project subcortically and form a major termination zone in the pontine nuclei of the brainstem which relays information to the cerebellar cortex and a smaller fraction of ponto-cerebellar axons also targets the cerebellum's motor output nuclei (Caligiore et al., 2016). These axon branches might carry prediction signals essential for updating cerebellar internal models for example by comparing sensory input impinging via the inferior olive with motor efference copies. An experiment which could shed light on the question if information conveyed by layer 5 PT neurons differs depending on the target could be recording at different sites of termination, for example in pontine nuclei or dorsal striatum.

Interestingly, in a human reaching task it was found that the sensory prediction error alone (without the actual motor correction) was sufficient for motor adaptation (Tseng et al., 2007). I speculate that for driving motor learning, mice do not actually need to execute the movement. This could explain why layer 5 PT neurons, although their activity is reminiscent of motor commands, do not actually need to drive movement in order to be essential for motor learning. It might also provide a rationale for why these neurons, the activity of which has generally been considered as motor commands, innervate many more sites throughout the brain than they do innervate actual motor effectors (Kita and Kita, 2012).

6.6.2.2 Activity during running

During self-initiated locomotion-onsets (**Figure 19**) I found in layer 2/3 neurons, similar to spontaneous turns (**Figure 18**), that activation is decreased as mice learned the task. In layer 5 PT neurons, albeit noisy, I found an increase in activation. Layer 5 IT neurons did not change their

responses.

This is in excellent agreement with the previously outlined predictive coding scheme (**Figure 30C**). Layer 2/3 neurons might represent error units signaling the deviation between predicted and intended change of the environment. As behavior becomes learned, this error decreases, hence activity in layer 2/3 should decrease, which is what I observed. Interestingly, this learning-related decrease in activation seemed to be mediated mainly by a large reduction in preparatory activity (**Figure 19C1**). This opens up the possibility that preparatory activity is not signifying a state of activity devoted to movement planning as suggested during primate instructed-delay studies. Rather, in a predictive coding interpretation, preparatory activity could signal the anticipated uncertainty in the internal model at the beginning of learning. This would suggest that the activity of error units would not only signal the deviation of predicted from actual state but part of their activity could signal an overall estimate of uncertainty of the environment.

If layer 5 PT neurons were prediction units, their activity should increase. Albeit noisy, I can observe such an increase, confirming the position of layer 5 PT neurons as prediction units within the predictive coding framework (Adams et al., 2013).

An interesting observation arose from comparing data obtained when mice were running in VR or in darkness (**Figure 19**). In running speed-matched comparisons I observed that:

1) Running-related activity in darkness was significantly higher than when running in VR.
2) During running in darkness, running-related activity did not change in a learning-dependent manner.

This is in good agreement with predictive coding schemes which posit that activity depends on the context and is situational (see *Claim 2* above). In particular, if layer 2/3 neuronal activity was representative of prediction errors, I would have expected such a pattern. In darkness, mice are deprived of visual feedback, thus the environment is highly uncertain to the mice. This makes the prediction meaningless and thereby produces many prediction errors which are reflected in high activity of presumptive layer 2/3 error units. Additionally, prolonged experience in darkness does not help finessing the internal model since the feedback about the success of mice's movement is seemingly random. Hence, activation in layer 2/3 does not change in a learning-related manner when mice run in darkness.

From human psychophysics it has been concluded that internal models derived from different senses can compensate for one another (Sainburg et al., 1993) and multiple internal models can co-exist and be learned in parallel (Krakauer et al., 1999). This raises the question if internal model-specific error signals exist which might map onto movement types. My data are agnostic to this assumption: Running and turning likely fall in grossly similar movement categories (e.g. both largely use the same muscles) and, more important, learning to navigate the VR will affect both internal models similarly, making it hard to experimentally distinguish either internal

model with this task. To address this question, I would train mice on two distinct tasks in which execution of behavior relies on different sensory modalities. This might allow assessing if the same neurons mediate distinct internal models or if the models map onto discrete cell populations.

6.6.3 Local sensorimotor transformation could enable comparisons across coordinate systems

Comparisons within neuronal circuits are sensible only when performed between alike entities. For example, for us, it might be easy to compare the personal preference of cucumber and tomatoes for making a salad but it's much less sensible to compare cucumbers and bananas for making a salad because those ingredients are not commonly found in one salad. Likewise, comparisons in neuronal circuits should happen within the same frame of reference: broadly speaking either motor or sensory frame of reference. Note however, that a division between "motor" and "sensory" domains in the brain is likely arbitrary because the common goal of the brain is to generate movement by virtue of its sensory input which implies that cortical circuits smoothly integrate information upon demand. This notion is also supported by the fact that the cortical layer architecture is largely homogenous over a wide range of areas, making it hard to definitely ascribe various regions to either domain. For simplicity of the argument, I will nevertheless refer to sensory and motor as the type of stimulus which best correlates with the activity encountered in a given brain area.

Input onto layer 2/3 neurons could either be in sensory or motor coordinates (**Figure 30**). Resolving this question is controversial: One line of support comes from the fact that motor cortex is agranular. In primary sensory areas, the thalamus conveys information from the sensory periphery to the granular layer (layer 4) of cortex. These inputs are by definition in sensory coordinates. Motor cortex, however, is lacking a distinct layer 4 (though for a different point of view, see Yamawaki et al., 2014). This could imply that input to local motor cortex is mainly in motor coordinates.

Alternatively, input to local motor cortex could be in sensory coordinates. Two observations make this hypothesis appealing: First, in humans using saccadic eye movements, it has been suggested that errors updating movement (the layer 2/3 units in my data) are obtained by a comparison of observed (actual) visual sensory error and a realistic estimate of movement outcome (prediction) (rather than the actual movement outcome) (Wong and Shelhamer, 2011). This would imply that both, the observed signal and the estimate are in sensory coordinates. In my model, layer 2/3 might mediate such a computation. Second, studies performed in primates suggest that motor cortex itself is the site of coordinate system transformation from a sensory frame of reference to a motor frame of reference implying inputs are in a sensory frame of

reference (Shen and Alexander, 1997a; Zhang et al., 1997). Note that the studies investigated different sensory modalities (spatial and visual, respectively), suggesting multiple streams of processing in motor cortex. Taken together, it seems conceivable that both inputs to layer 2/3 neurons, the actual movement and the prediction of movement outcome, are compared in sensory coordinates.

Layer 2/3 outputs an error signal which contains information of how the motor command should be updated (**Figure 30**, the motor plan). The intracolumnar connectivity between layer 2/3 and layer 5 PT neurons could then serve as the substrate of a sensorimotor transformation which generates motor command-like activity in layer 5 PT neurons.

6.6.4 Origin of signals for internal model learning

Classic reinforcement learning theories require error signals which provide a measure of difference between desired and actual state to drive learning. Motor adaptation similarly has to rely on error signals to incrementally optimize behavior. Adaptations in saccadic eye movements in humans were suggested to rely on expectations about movement outcomes (rather than actual outcomes)(Wong and Shelhamer, 2011). Difference signals between expected and observed outcome of a movement could lie at the heart of motor learning. My data suggest that such a comparison might take place in motor cortical circuits.

Sensory prediction error is important for fine-tuning motor behavior and its on-line control (Berniker and Kording, 2008; Todorov and Jordan, 2002) and deep cerebellar nuclei have been shown to accurately track sensory prediction errors depending on the probability of their occurrence (Brooks et al., 2015). Thus, sensory predictions which are suggested as a major input in my model for motor cortex (**Figure 30**) could be derived by cerebellar processing routed via the cerebellar-thalamo-cortical loop (see Introduction) to local motor cortex. These signals have also been shown to drive motor adaptation and motor learning (Tseng et al., 2007).

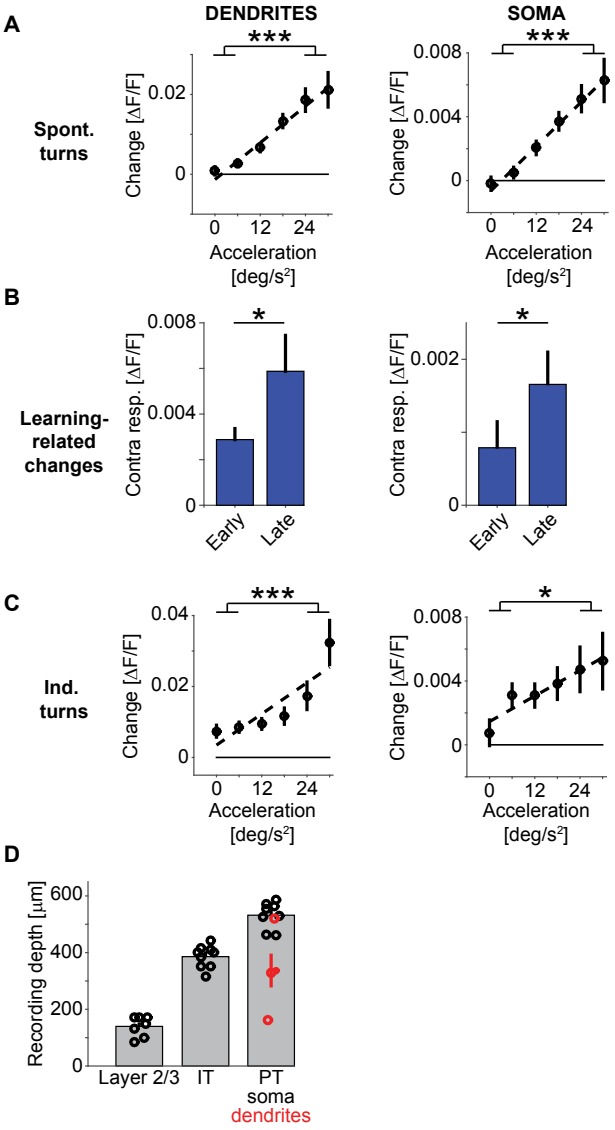
Credit assignment is a fundamental problem for the brain: In all behavioral contexts, the impact of the action on the behavioral goal has to be estimated and advantageous actions should be recognized and memorized for repeated execution in future instances of the same context. Somehow, the brain needs to decide what has been a good action and what has been a disadvantageous action and the former need to be reinforced over the latter.

Neuromodulators are prominent candidates to mediate this reinforcement. Ever since activity of dopaminergic neurons has been observed to correlate with reward prediction errors (Hollerman and Schultz, 1998), it became clear that their activity could mediate the teaching signal required in various models of learning (Bush and Mosteller, 1951; Suri, 2002; Sutton, 1988). Subsequently, phasic dopaminergic activity was demonstrated to reflect reward prediction errors

important for learning (Steinberg et al., 2013). In rat motor cortex, dopaminergic projections from the VTA have been described and found to be important for reaching motor skill learning (Hosp et al., 2011) but not execution (Molina-Luna et al., 2009).

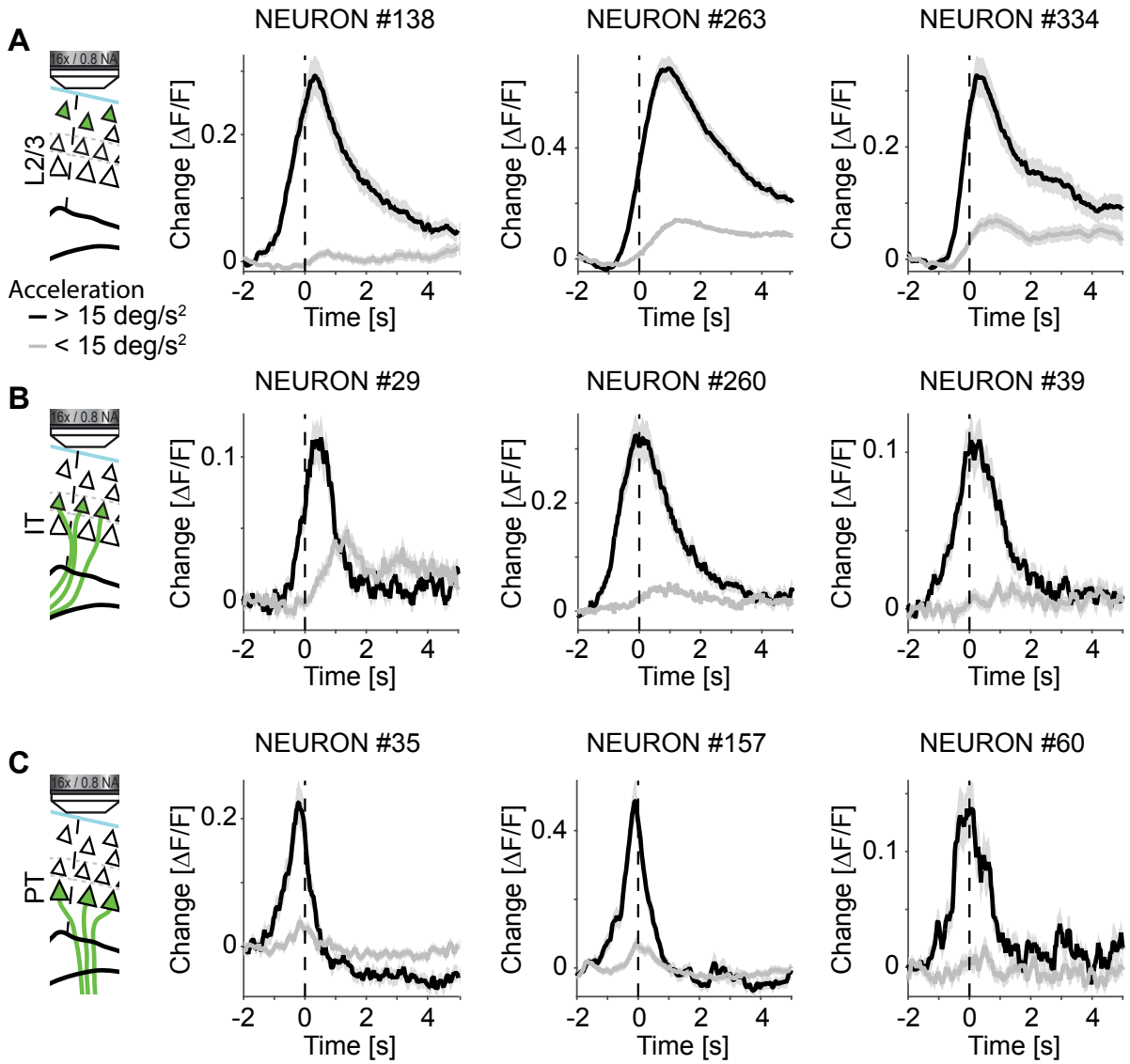
Based on the predictive coding framework, it is possible that dopamine in local motor cortex circuitry could reinforce the updating of internal models represented in the activity in layer 5 projection neurons. Increase in LTP has been a demonstrated capability of local dopamine signaling motor cortex (Molina-Luna et al., 2009). Thus, the coincident arrival of phasic dopaminergic activity together with inputs from layer 2/3 neurons and other sources could lead to strengthening of the synaptic weights of these connections in the local circuits.

8. SUPPLEMENT



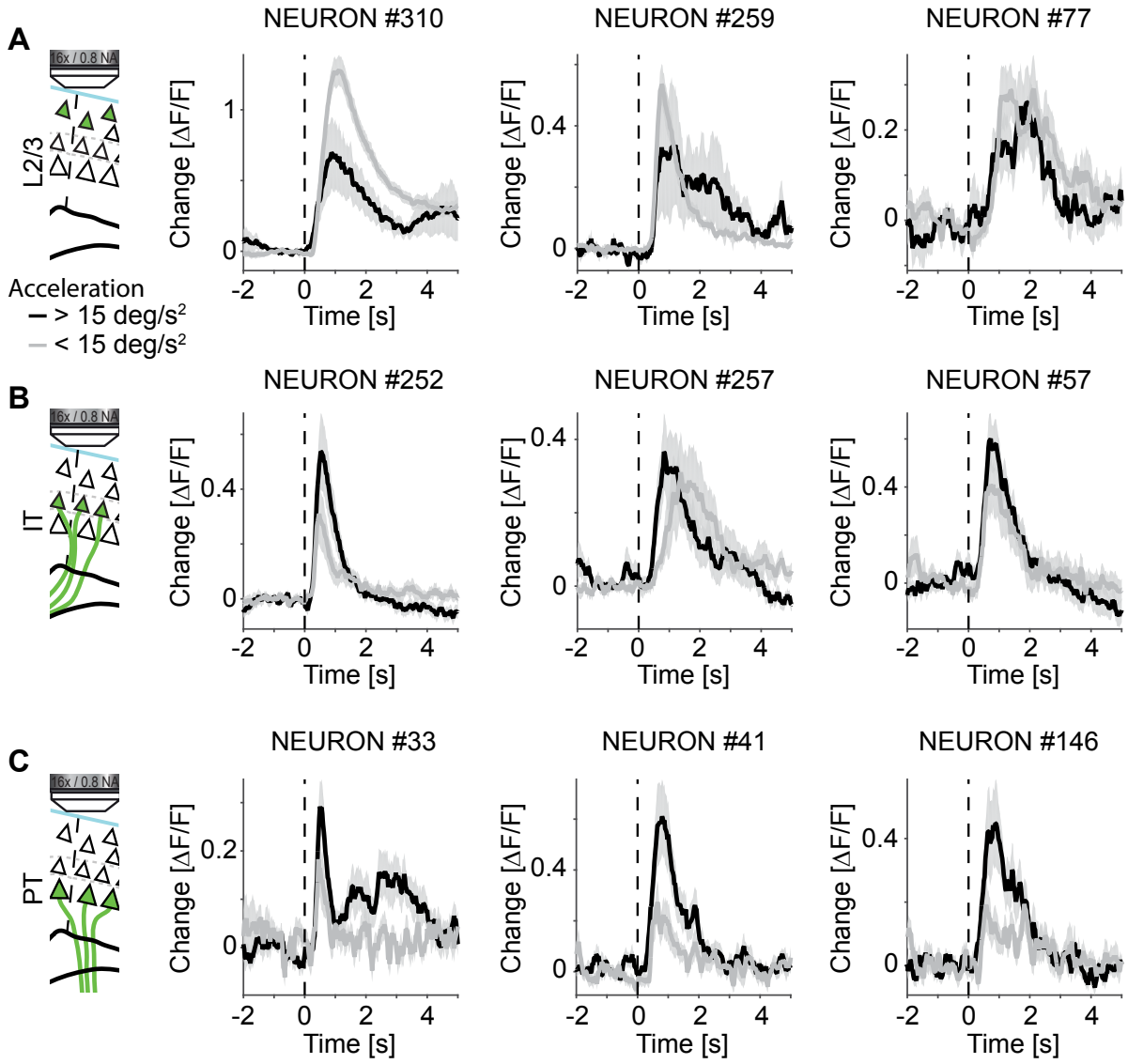
Supplementary Figure 1. Pyramidal tract dendrites and soma have qualitatively similar response properties

(**A**) Average activation during spontaneous turns binned to different acceleration for layer 5 PT dendrites (left panel) and layer 5 PT soma (right panel). Dashed black line indicates a line fit to the data. Solid black line marks 0 [ΔF/F]. Error bars are SEM over entities (n = 336, 224 dendrites and soma, respectively). ***, $p < 0.0005$. (**B**) Average response of layer 5 PT dendrites (left panel) and soma (right panel) to contraversive spontaneous turn events over the course of learning. Error bars are SEM over entities. *, $p < 0.05$. (**C**) Average activation during induced turns binned to different acceleration for layer 5 PT dendrites (left panel) and layer 5 PT soma (right panel). Dashed black line indicates a line fit to the data. Solid black line marks 0 [ΔF/F]. Error bars are SEM over entities (n = dendrites and soma, respectively). *, ***, $p < 0.05$, 0.0005. (**D**) Average recording depth for either cell type. Open squares indicate values for individual mice (layer 2/3, IT and PT, n = 7, 8, 11). PT in red are dendritic recording sites. Error bars indicate SEM over mice.



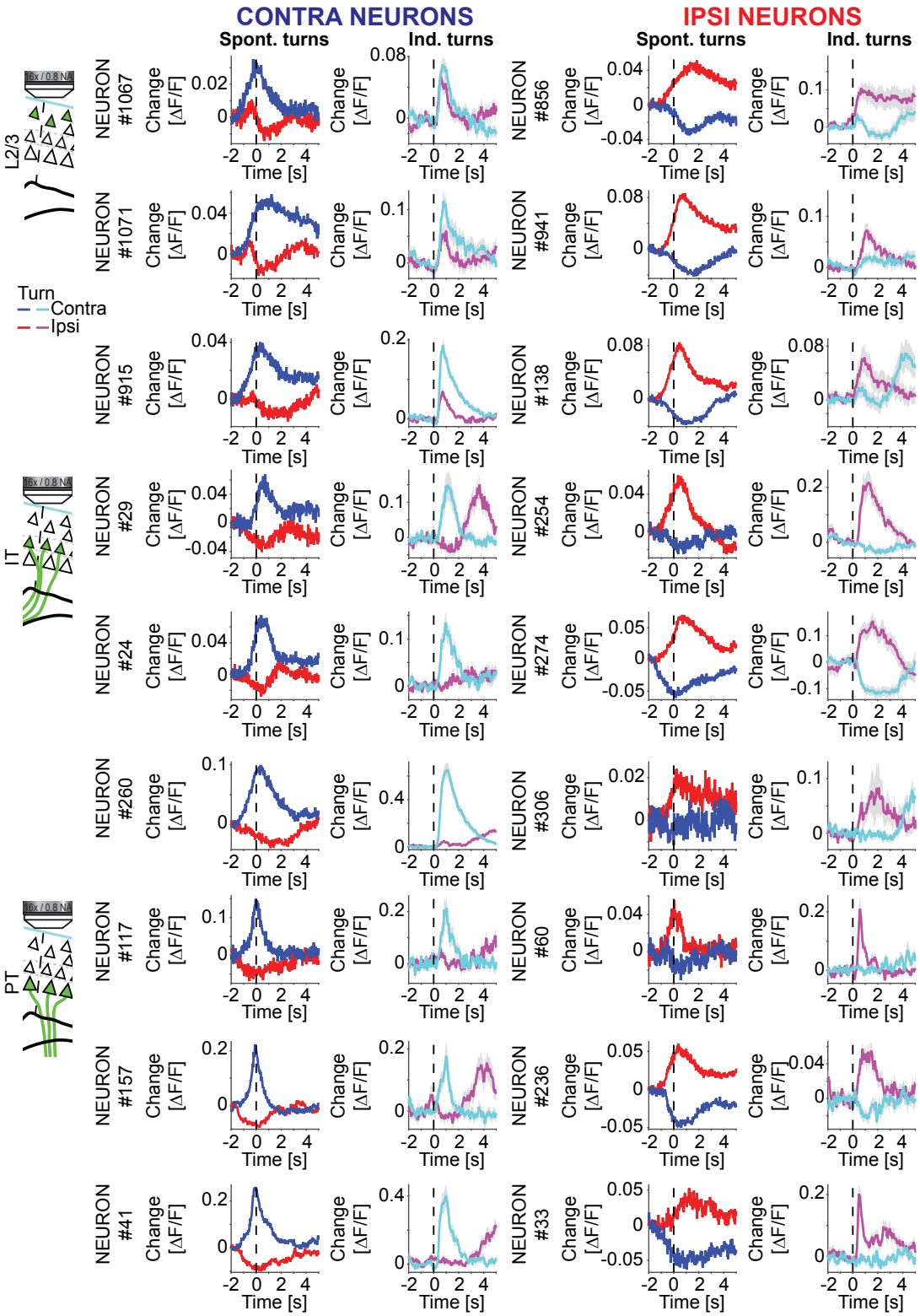
Supplementary Figure 2. Example neuronal responses during spontaneous turns of different acceleration

(**A**) Schematics of recording in layer 2/3 (left panel) and individual example neuronal average activity during spontaneous turns of low (grey) or high (black) acceleration. (**B**) Same as (**A**) but for layer 5 IT neurons. (**C**) Same as (**A**) but for layer 5 PT neurons.



Supplementary Figure 3. Example neuronal responses during induced turns of different acceleration

(**A**) Schematics of recording in layer 2/3 (left panel) and individual example neuron's average activity during induced turns of low (grey) or high (black) acceleration. (**B**) Same as (**A**) but for layer 5 IT neurons. (**C**) Same as (**A**) but for layer 5 PT neurons.



Supplementary Figure 4. Example neuronal responses during contra- and ipsiversive spontaneous and induced turns

For the three cell types (indicated in the left column) example neuronal activation during spontaneous turns, either contra- (blue) or ipsiversive (red), and the same neuron's activation during induced turns, either contra- (cyan) or ipsiversive (magenta). Time zero marks onset of spontaneous turn or onset of visual offset perturbation stimulus.

7. CLOSING REMARKS

The importance of studying motor control, the way movements are generated, can hardly be overstated as movement is the fundamental interaction with the world. The degree to which this control is achieved and how is an embattled question if nothing else because medical efforts in neurological conditions of movement apparatus dysfunction would profit from insight.

I hypothesized that sensory guidance is a key factor impacting high-level motor control and designed a task in which I could distinguish motor control mechanisms during ongoing, expected (spontaneous) behavior from sensory-guided behavior in reaction to unexpected perturbations of the environment. I found, using optogenetic activity perturbations, that ongoing motor cortical activity is essential for initiation and execution of appropriate, visually guided motor programs. Moreover, recordings showed that during spontaneous and forcefully induced visually guided behavior, patterns of neuronal activity differed, providing an explanation for the previously observed behavioral phenotypes. In summary, I found that normal motor cortex activity was critical for the appropriate execution of visually guided behavior.

These findings might extend to other sensory modalities or sensory guided behavior in general, perhaps providing a principled explanation for different phenotypes of motor cortex lesions. Species which heavily rely on their senses and in particular those senses sampling the distal environment such as vision, might require much more involvement of motor cortex as many internal models have to be processed simultaneously for successful behavior. Consequently, damage to motor cortical areas has a much bigger impact in those species such as, for example, us humans.

I therefore think that the study of motor control mechanisms during sensory guided behavior could, in the long run, provide explanations for the degree of sophistication of human brains that is unmatched by any other species.

9. EXPERIMENTAL PROCEDURES

Mice. All animal procedures were approved by and carried out in accordance with guidelines of the Veterinary Department of the Canton Basel-Stadt, Switzerland. The mice used in this study were the following: 8 *vGAT-Cre* (Vong et al., 2011, JAX #016962) x *ROSA-LSL-tdTom* (Madisen et al., 2010, JAX #007914) mice were used for imaging layer 2/3 excitatory neurons, 11 *Sim1-Cre(KJ18)* mice for imaging layer 5 PT neurons (Gerfen et al., 2013, MMRRC #031742), 6 *vGAT::ChR2(H134R)-EYFP* (JAX #014548) mice were used for optogenetic inhibition experiments and 6 *C57BL/6* mice were used in optogenetic control experiments. Mice were group housed in a vivarium (light/dark cycle: 12/12 hours) and were 6 – 14 weeks old at the beginning of experiments. Experimental mice used were of both sexes.

Surgery and virus injections. For all surgical procedures, mice were anesthetized using a mixture of Fentanyl (0.05 mg/kg; Actavis), Midazolam (5.0 mg/kg; Dormicum, Roche) and Medetomidine (0.5 mg/kg; Domitor, Orion). In surgeries preparing for two-photon imaging experiments, a craniotomy of approximately 4 mm diameter was made over the right motor cortex centered on a location 0.5 mm anterior and 1.5 mm lateral of bregma. This corresponds approximately to the caudal forelimb area mapped by intracortical microstimulation (Tennant et al., 2011). We placed 5 to 9 injections of approximately 150 nl each of either unconditional AAV2/1-Ef1α-GCaMP6f (titer 10^{11} – 10^{12} GC/ml) for imaging layer 2/3 neurons or conditional AAV2/1-Ef1α-DIO-GCaMP6f (titer 10^{11} – 10^{12} GC/ml) for imaging layer 5 PT neurons within a radius of approximately 500 µm of the center of the target region. For optogenetic experiments, the craniotomy was made to span motor cortex bilaterally. A circular 4 mm (imaging) or a custom-cut (optogenetics) coverslip was implanted with superglue (Pattex) to close the craniotomy. A custom-machined titanium head bar was attached to the skull using dental cement (Paladur, Heraeus). Anesthesia was antagonized by an intraperitoneal injection of a mixture of Flumazenil (0.5 mg/kg; Anexate, Roche) and Atipamezole (2.5 mg/kg; Antisedan, Orion Pharma) and mice were returned to their home cage with access to a running wheel and water ad libitum for at least 3 days before beginning of the experiment.

Virtual reality setup and behavioral paradigm. For all experiments we used a virtual reality setup as previously described (Leinweber et al., 2014). Mice were head-fixed and free to run on a spherical, air-supported styrofoam ball (**Figure 4**). Rotation of the ball was coupled to movement in a virtual reality environment. A virtual corridor was projected onto a toroidal screen positioned in front of the mice covering approximately 240 degrees horizontally and 100 degrees vertically of the field of view using a projector (Samsung SP-F10M). To prevent light leak from the projector onto the two-photon imaging, the projector was synchronized to the resonant scanner of the microscope. Mice were trained to control heading in the virtual tunnel and navigate towards a target. Upon reaching the target, a reward was delivered through a blunt syringe spout positioned

near the snout of the mouse (~ 7 µl / reward, 1:10 diluted milk). To incentivize mice to engage in the training paradigm, they were water-restricted with access to 1 ml water daily 3 days before start of either imaging or optogenetic experiments. Care was taken to prevent a drop in body weight to below 80% of starting weight and additional water was supplemented when necessary. The amount of rewards the mice could collect during training was not restricted. A new trial was initiated after a 5 s time out during which a gray screen was presented. The tunnel was kept short initially (length to width ratio of 5:1) but was progressively lengthened to increase difficulty of the task such that mice collected approximately 2 rewards per minute. By the end of training, the tunnel length typically corresponded to approximately 4 m of physical distance (length to width ration of 30:1). Mice were trained and imaged daily in training sessions that lasted approximately 45 minutes. From training session three onwards, visual offset perturbations were introduced with a probability of 0.8 per traversal at a random location in the virtual tunnel (restricted to the middle portion of the tunnel between 20% and 80% of total length). Visual offset perturbations consisted of a sudden 30° offset in the heading randomly either to the left or to the right.

Optogenetic inhibition experiments. Mice were trained to navigate the virtual environment as described above. Light from a 473 nm laser (Shanghai Laser & Optics Century, BL473H-200) was sinusoidally-modulated at 40 Hz and average laser power was adjusted to 45 mW / mm^2 using a Pockels Cell (Conoptics, Model 350-80). Full-width half maximum of the laser beam was 0.8 mm and power dropped to 1/e at a radius of 0.5 mm. Photostimulation was performed through a cranial window. We used a galvo-galvo scanhead to direct the laser beam at one of four (two per hemisphere) target locations in forelimb motor cortex (0.5 mm rostral, 1.5 mm lateral and 1.5 mm rostral, 1.5 mm lateral from bregma) and two blank locations on the head bar. For stimulation, the laser cycled between each of the four target locations for the duration of the stimulus with a dwell time of 20 ms per location. To minimize stimulation related changes in noise generated by the moving galvanometers and luminance changes associated with scanning the laser beam, the laser was continuously cycled between the two blank locations on the head bar during times of no stimulation. For the chronic inhibition experiments during training (**Figures 6 and 9**), we stopped inhibition of motor cortex in cases where the trial lasted more than 15 s by cycling the laser between the blank positions. Inhibition was then resumed at the start of the next trial. This was done to increase the motivation of the mice to run on the spherical treadmill. For the experiments on the effect of motor cortex inhibition on the response to visual offset perturbations in trained mice (**Figures 7 - 9**), the laser was switched from blank to target position at different times relative to the onset of the visual offset perturbation (-1 s, -0.5 s, 0 s, 0.5 s, 1s). Different laser onset times were randomized and data were collected over four training sessions on four consecutive days.

Two-photon calcium imaging. All two-photon imaging experiments were performed using a modified Thorlabs B-Scope as described previously (Leinweber et al., 2014). Light source was a

femtosecond laser (MaiTai eHP DeepSee, Spectra Physics) tuned to 990 nm. The scan head was based on an 8 kHz resonant scanner (Cambridge Technology). For imaging we used a 16x, 0.8 NA objective (Nikon). Emission light was band pass filtered (525/50, Semrock BrightLine) and detected using GaAsP photomultiplier tubes (H7422, Hamamatsu). Photomultiplier tube signals were amplified (DHPCA-100, Femto), digitized (NI5772, National Instruments) at 800 MHz and band-pass filtered at 80 MHz using a digital Fourier-transform filter implemented in custom written software on an FPGA (NI5772, National Instruments). Images were acquired at 40 Hz frame rate at a resolution of 750 x 400 pixels.

Data analysis. All data analysis was performed using custom-written and publicly available (https://sourceforge.net/projects/iris-scanning) software written in MATLAB (MathWorks). Statistical tests were used as stated in the figure legends.

Behavioral data analyses. Onsets of spontaneous turns were determined by thresholding (0.12 deg/s) the absolute value of rotational velocity of the spherical treadmill. To isolate well-defined turns, turn onsets that were preceded by another turn in the same direction within 5 s were discarded. This excluded 45% of the turns identified with the threshold crossing criterion. This procedure was aimed at eliminating threshold crossings that occurred in rapid succession either as a result of noise in the recording or as small rotational velocity changes due to individual steps. Results did not change qualitatively when varying this exclusion window size over a wide range (2 s - 10 s). Onsets of running and fraction of time spent running during a training session were determined by thresholding the forward velocity of the spherical treadmill (1.2 cm/s). To isolate running onsets, running onsets were discarded for analysis that were preceded by running in a window of 2.5 s or were within 5 s of a reward delivery.

To estimate the time to initiation of a turn (**Figure 5**), a vector of induced turn behavioral responses was calculated in bins of 25 ms, from 0 s to +1.125 s after the visual offset. Repeated Student's t-tests were used versus a baseline vector obtained 25 ms prior to visual perturbation onset. The first statistically statistically significant comparison was considered onset of behavior. Acceleration was estimated as the slope of a line fit from onset of visual perturbation to maximum speed of turning.

Performance (**Figure 6 and 9**) was quantified as the fraction of time spent running towards the target (± 36 degrees from the direction of the target) normalized by the total time spent running. The learning rate was calculated as the slope of a line fit to the performance data (**Figure 6**). Rotational acceleration during a turn was approximated as the average slope of the velocity curve between the onset of visual offset perturbation and the time of maximum speed (**Figure 5**). For the visual offset perturbation responses, delayed response trials were defined as trials in which turning velocity of the mice did not cross a threshold (10 deg/s) within 1 s after visual offset perturbation (**Figure 7 - 9**).

Calcium imaging data analyses. Raw two-photon images were full-frame registered to correct for brain motion. Neurons were manually selected based on mean and maximum fluorescence images. Note that the use of maximum fluorescence images biased the selection of neurons to active neurons. Raw fluorescence traces were computed as the mean fluorescence of the image pixels corresponding to one neuron. Fluorescence time series were corrected for slow drift in brightness using an 8^{th}-percentile filtering with a 15 s-window as described previously (Dombeck et al., 2007). $(F-F_0)/F_0$ (abbreviated as ΔF/F) values were calculated on the drift corrected raw fluorescence values (F) using the median fluorescence (over the entire recording session) for normalization (F_0).

To compute the average event-triggered change in fluorescence ΔF/F, responses of each neuron were baseline subtracted using the baseline windows: for spontaneous turns -2 s to -1.5 s; for visual offset perturbation induced turns and running onset -0.5 s to -0 s, for reward delivery -5 s : -4.5 s, texture change (mid-point of tunnel) -1.125 s : -0.625 s, visual cue onset (5 s after reward delivery) -1.125 s : -0.625 s and preparatory activity -2.5 s : 2 s preceding the running onset. To compute average responses, differences of mean ΔF/F in a response window and mean ΔF/F in the baseline window were used. Response windows were: for spontaneous turn responses: 0 s to 1 s; for visual offset perturbation induced turns and running onset response: 0.5 s to 1.5 s, reward delivery pre-response window - 0.5 s : 0 s and post-response window 0 s : 0.5 s, texture change 0.25 s : 1.25 s, visual cue onset 0 s : 1 s and preparatory activity - 1 s : 0 s preceding the running onset.

Contraversive (ipsiversive) turn neurons were defined if their response to contraversive (ipsiversive) turns was stronger than their response to ipsiversive (contraversive) turns. To obtain a population response, the average of the activity of contraversive turn neurons to contraversive turns and the average of ipsiversive turn neurons to ipsiversive turns was calculated, and these two responses were then averaged.

Linear trend analysis was performed using the Matlab regress function. To quantify the significance of the linear trend, the p-value of the F statistic is reported.

To speed-match events (**Figure 18 and 19**), high speed trials were omitted based on thresholding. The threshold was chosen such that statistical significance was not reached in pairwise tests for same mean peak values for all conditions ($p > 0.05$, unpaired Student's t-test). Neuronal activity was then aligned to the respective remaining events.

Cellular responses (**Figure 21**) were computed for either turning events in the respective analysis windows. Colors were assigned according to the selectivity of the cells' response during spontaneous turns determined as Selectivity = ΔResponse(contraversive - ipsiversive). Selectivity > 0 were assigned blue hues and selectivity < 0 were assigned red hues.

The time course of population vector correlation (**Figure 22**) was obtained by calculating the average Pearson's correlation of the population vector of activity during ipsi- and contraversive turns for all mice. The population vector of activity was calculated as a function of time, averaging the activity in a window of 0.4 s in steps of 0.2 s. To account for differences in the number of turns per mouse, an equal number of turns was randomly subselected (corresponding to number of turns from the mouse with the least turns) from all mice. To obtain estimates of the baseline level of correlation, the population vector of activity correlation at random times during running was computed.

To visualize the dynamics of the population vector of activity around spontaneous and induced turn onsets (**Figure 23**), the population vector of activity for each turn event was projected, calculated in time bins of 0.1 s, onto the two-dimensional space spanned by the population vector for a spontaneous right turn and the population vector for a spontaneous left turn (calculated as the difference between 0 s and 1 s after turn onset). This was done for spontaneous contra- and ipsiversive, and induced contra- and ipsiversive turns separately. For this analysis, data from mice with less than 15% contraversive or ipsiversive neurons were excluded (0 of 8 layer 2/3 mice and 2 of the 11 layer 5 PT mice), as in these mice the estimate of spontaneous turn basis vectors was too noisy. The onsets of the behavioral response for the induced turns (Figure 8, black crosses) were estimated as the time bin with first significant ($p < 0.05$, Student's t-test) deviation of the distribution of rotational velocity after the visual offset perturbation (in a window of 25 ms, measured in steps of 25 ms) from the distribution immediately preceding the visual offset perturbation (in a window of 25 ms).

The time mice passed the mid of the length of the virtual tunnel was used as the time point of texture change (**Figure 25 and 26**).

The time 5 s after reward delivery was defined as the time point of visual cue onset (**Figure 27 and 28**).

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11. LIST OF ABBREVIATIONS

AAV – Adeno-associated virus

ALM – Anterior-lateral motor (cortex)

BMI – Brain-machine interfaces

CCD – Charge-coupled device

CPG – Central pattern generator

CST – Cortico-spinal tract

CT – Cortico-thalamic

EMG – Electromyography

FOF – Frontal orienting field

GABA – γ-aminobutyric acid

ICMS – Intracortical microstimulation

IT – Intra-telencephalic

LRN - Lateral reticular nucleus

LTP – Long-term potentiation

M1 – Primary motor cortex

M2 – Secondary motor cortex

MSN – Medium spinal neuron

PD – Parkinson's Disease

PFC – Prefrontal cortex

PM – Premotor cortex

PPC – Posterior parietal cortex

PT – Pyramidal tract

S1 – Primary somatosensory cortex

S2 – Secondary somatosensory cortex

SN – Substantia nigra

SOM – Somatostatin

TD – Temporal difference

TMS – Transcranial magnetic stimulation

V1 – Primary visual cortex

VR – Virtual reality

VTA – Ventral tegmental area